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The potential application of Trichoplusia ni granulovirus En3 enhancin as a synergist in baculovirus-based insecticides.

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The potential application of Trichoplusia ni granulovirus En3 enhancin as a synergist in baculovirus-based insecticides.

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que la presente memoria de Tesis Doctoral titulada "**The potential application of Trichoplusia ni granulovirus En3 enhancin as a synergist in baculovirus-based insecticides.**" elaborada por Dña. **ADRIANA RICARTE BERMEJO** ha sido realizada bajo nuestra dirección, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona a 2 de julio de 2021,

Prof. Dr. Primitivo Caballero Murillo Dra. Oihane Simón de Goñi

TABLE OF CONTENTS

RESUMEN

Los grandes costes asociados a la producción masiva de baculovirus hacen necesaria la búsqueda de productos sinérgicos que reduzcan la cantidad de materia activa. En la presente tesis, se expresó y produjo un factor sinérgico con gran potencial para formulaciones basadas en baculovirus utilizando un sistema de expresión de baculovirus.

Los agentes disruptores de la matriz peritrófica aumentan la susceptibilidad de las larvas de lepidópteros a la infección por baculovirus. Se realizó una extensa revisión de la literatura existente sobre las sustancias que degradan la MP, tales como abrillantadores ópticos, enhancinas virales y bacterianas y quitinasas, con el fin de seleccionar el mejor factor potenciador para su producción en un vector de baculovirus. Los abrillantadores ópticos produjeron, con mucho, el mayor incremento de la infección por baculovirus en condiciones de laboratorio. Por ejemplo, algunos de ellos reducen hasta 5000 veces la CL_{50} del NPV de *Spodoptera frugiperda* contra su huésped homólogo. Sin embargo, el efecto de potenciación en campo nunca alcanza los datos obtenidos en condiciones de laboratorio. Además, su alto coste y su influencia negativa sobre los cultivos, la polarización y el medio ambiente los hacen inaceptables para las formulaciones de baculovirus. Además, su origen químico no se considera en sintonía con los productos de control biológico de plagas. Por el contrario, las enhancinas y quitinasas de origen natural que poseen actividad proteasa para degradar la PM también facilitan la infección inicial del virus. Hasta ahora, solo un estudio informó un aumento de la infección por baculovirus, hasta 5,4 veces, al agregar una quitinasa comercial. Por el contrario, las enhancinas se han estudiado ampliamente como potenciadores de las infecciones por baculovirus, utilizando diferentes enfoques. Las enhancinas de baculovirus presentes en granulovirus y nucleopoliedrovirus degradan la PM, pero de manera diferente. Las enhancinas de granulovirus (GV) están presentes en los OB y degradan las MP de forma aleatoria. Las enhancinas de nucleopoliedrovirus (NPV) tienen acceso a las células del intestino medio formando un túnel en la MP, siendo su impacto en la MP más sutil. Las diferencias entre las enhancinas de diferentes betabaculovirus, las enhancinas de TnGV han sido, con mucho, las más estudiadas y mostraron la mayor potenciación de la infección. El genoma de TnGV posee tres potenciadores: En3

posee el dominio de unión a Zinc y el dominio de unión a mucina característico, importante para conferir las propiedades de las proteinasas, mientras que En1 y En 4 carecen de ambos motivos. Por todas las razones anteriores, se eligió la En3 para su posterior experimentación.

Se construyó un AcMNPV recombinante positivo para polihedrina que expresaba la En3 de TnGV. La En3 recombinante se produjo con éxito y se acumulaba en las células infectadas, específicamente en la fracción soluble de las células tras la lisis, y no en los OB ni en los ODV. La fracción soluble fue responsable de un aumento en la patogenicidad de las infecciones por NPV. Por ejemplo, aumentó las infecciones por AcMNPV en aproximadamente 3 veces en el segundo estadio y en 4,7 veces en las larvas del cuarto estadio de *S. exigua*. Estos resultados revelaron un efecto de estadio, más intenso en infecciones de estadios más maduros, e indicaron que la producción de enhancinas solubilizadas usando sistemas de expresión basados en baculovirus puede usarse para producir OB de baculovirus con una eficacia mejorada.

Finalmente, se llevó a cabo una producción a escala de laboratorio de la proteína En3 para evaluar la viabilidad de su producción en masa. La producción se logró *in vitro* e *in vivo.* Ambos sistemas podrían utilizarse eficazmente para este propósito. Las enhancinas solubilizadas en células de insectos y la fracción soluble de cultivo celular pueden aumentar la patogenicidad del baculovirus cuando se añaden a diferentes virus-huésped y se pueden usar en combinaciones con OB de baculovirus. Sin embargo, el sistema *in vitro* es más costoso. Técnicamente, se trata de rondas seriadas de centrifugación para eliminar las diferentes fases para obtener la enhancina solubilizada, que posteriormente se debe purificar a través de filtros de 0,2 µm para eliminar los virus no-ocluidos presentes en el medio celular. Además, en la producción *in vitro*, el volumen final de sustancia potenciadora recuperada es más bajo, de 500 µl/flask de 75 cm^2. Por el contrario, el sistema *in vivo* fue más factible ya que se requirió menos manipulación y permitió obtener mayores volúmenes (10 mL/10 larvas). El sistema *in vivo* implicó la infección de especies en estadio tardío altamente susceptibles, como larvas de *S. exigua* o *T. ni*, recogidas antes de su licuefacción, y posterior incubación controlada para permitir la

licuefacción total de las larvas. Finalmente, se llevó a cabo la purificación de la fracción soluble de las células mediante una minuciosa filtración por muselina.

Los bioensayos permitieron determinar la concentración mínima de sustancia potenciadora necesaria para mejorar las infecciones por baculovirus, que se estableció en 100 ng/µl. A esta concentración, la fracción soluble fue capaz de potenciar la actividad de AcMNPV en su especie heteróloga *S. exigua* en 4,2 veces, y en su especie homólogos *S. exigua*-SeMNPV en 15 veces, y *S. littoralis*-SpliNPV en 11 veces. Usando 10 larvas de *T. ni* o *S. exigua* con el sistema *in vivo*, se obtuvieron 10 ml a 2000 ng/ µl de sustancia potenciadora. Esto implica que sólo se necesitarían 50 larvas para producir la cantidad de sustancia potenciadora necesaria para tratar 1 Ha (1 litro de producto).

El principal logro de la presente tesis es que la producción *in vivo* de sobrenadante con enhancinas utilizando sistemas de expresión basados en baculovirus puede utilizarse para mejorar la eficacia de los insecticidas biológicos contra plagas de lepidópteros, reduciendo la materia activa de los bioinsecticidas y haciéndolos competitivos comercialmente en comparación con los productos químicos.

SUMMARY

The increased costs associated with baculovirus mass-production urge the search for synergistic products that reduce the amount of active matter. In the present thesis, a synergistic factor with great potential for baculovirus-based formulations was expressed and produced using a baculovirus expression system.

Peritrophic matrix disrupting agents increase the susceptibility of lepidopteran larvae to baculovirus infection. An extensive review on the literature covering compounds that degrade the PM such as optical brighteners, viral and bacterial enhancins, and chitinases was performed to select the enhancing factor for its production in a baculovirus vector. Optical brighteners produced by far the highest enhancement of baculovirus infection under laboratory conditions. For example, some of them reduce up to 5000-fold the LC_{50} of Spodoptera frugiperda NPV against its homologous host. However, the potentiation effect in the field never reaches those reported in the laboratory. Moreover, their high cost and negative influence on crops, polarization and the environment render them unacceptable for baculovirus formulations. Moreover, their chemical origin is not considered rational in biological control products. In contrast, naturally occurring enhancins and chitinases with protease activity for degrading the PM also facilitate the initial virus infection. So far, only one study reported an enhancement of baculovirus infection, up to 5.4-fold, by adding a commercial chitinase. In contrast, enhancins have been extensively studied as enhancers of baculovirus infections using different approaches. Baculovirus enhancins found in granuloviruses and nucleopolyhedroviruses both degrade the PM but differently. Granulovirus (GV) enhancins are present in the OBs and degrade the PM in a random manner. Nucleopolyhedrovirus (NPV) enhancins gain access to midgut cells by forming a tunnel in the PM, being their impact in the PM more subtle. Among the different betabaculovirus enhancin species, TnGV enhancins have been, by far, the the most widely studied and showed the highest enhancement of infection. TnGV genome possess three enhancins: En3 has the characteristic zinc-binding and mucin-binding domain, important in conferring proteinase properties, while En1 and En 4 lack both motifs and the zinc-binding motif, respectively. For all the above reasons, En3 was chosen for further experimentation.

A polyhedrin positive recombinant AcMNPV expressing TnGV En3 was constructed. The recombinant En3 was successfully produced, and En3 accumulated in infected cells, specifically in the soluble fraction of the cells following lysis, and not in the OBs or ODVs. The soluble fraction was responsible for an increase in pathogenicity of NPV infections and not the OBs. For example, it increased AcMNPV infections by approximately 3-fold in second instars and by 4.7-fold in fourth instar larvae of *S. exigua.* These results revealed an instar effect, more intense in infections of older instars, and indicated that production of solubilized enhancins using baculovirus-based expression systems can be used to produce baculovirus OBs with improved efficacy.

A lab-scale production of En3 was carried out to evaluate the feasibility of its mass-production. The production was accomplished *in vitro* and *in vivo*. Both systems could be effectively used for this purpose as enhancins solubilized in insect cells and cell soluble fraction increased baculovirus pathogenicity when added to different virus-host combinations. However, the *in vitro* system was more arduous. Technically, it involved serial rounds of centrifugations to obtain the solubilized enhancin, which had to be passed through 0.2 µm filters to eliminate non-occluded viruses present in the cell medium. Also, *in vitro* production yielded low volumes, of 500 µl/75 cm^2 flask. In contrast, the *in vivo* system was more feasible, less labor-intensive and yielded higher volumes (10 mL/10 larvae). The *in vivo* system implied the infection of highly susceptible late instar species, such as *S. exigua* or *T. ni*, larvae, harvesting before larvae liquefied, controlled incubation to permit total liquefaction of larvae, and purification of the cell soluble fraction by filtration through muslin and centrifugation.

Bioassays allowed to determine the minimum concentration needed to enhance baculovirus infections, which was set at 100 ng/µl. At this concentration, the soluble fraction increased AcMNPV activity in the heterologous *S. exigua* by 4.2-fold, and the homologous systems *S. exigua*-SeMNPV by 15-fold and *S. littoralis*-SpliNPV by 11-fold. Using the *in vivo* system, 10 ml at 2000 ng/µl of enhancer substance was obtained using 10 *T. ni* or *S. exigua* larvae. This implies that only 50 larvae would be necessary to produce the quantity of enhancer substance necessary to treat 1 Ha (1L product).

The main achievement of the present thesis is that the *in vivo* production of solubilized enhancins using baculovirus-based expression systems can be used to improve the efficacy of biological insecticides against lepidopteran pests, reducing the active matter of bioinsecticides and making them commercially competitive with chemicals.

CHAPTER I

Introduction

1. GENERAL INTRODUCTION

Chemicals insecticides have contributed to an increased agricultural production by protecting crops from pests at a relatively low cost. However their indiscriminate use has caused numerous problems, including contamination of the environment, reduction of the ozone layer, development of resistance in target pests, reduction of non-target populations, and impact on human health (Hamblin et al., 1990; Casida and Quistad, 1998; Al-Zaidi et al., 2011; Pirsaheb et al., 2015). The increasing awareness of these hazards urged politicians to elaborate a rational legislation (EU Directive 2009/128/EC) for chemical pesticides use, and scientists to develop new safe control products (Nicolopoulou-Stamati et al., 2016). The two approaches complement each other as current regulatory restrictions on the use of chemical pesticides have renewed interest in the commercialization of novel pest control products, in particular bioinsecticides.

Insect pathogenic viruses, predominantly those belonging to the Baculoviridae family, have proved effective bioinsecticides against insect pests in different agronomical contexts. Baculoviruses cause fatal disease in more than 700 insect species, the vast majority of them in the orders Lepidoptera, Hymenoptera and Diptera (Herniou et al., 2003; Jehle et al., 2006). They are highly specific, with some members infectious to only one or two insect species (Caballero et al., 2009; Moscardi, 1999; Rodgers, 1993). Consequently, they are safe for vertebrates, humans and non-target organisms, and contribute to maintain biodiversity in agroecosystems (Ahmad et al., 2011; Ashour et al., 2007; Gröner, 1986). In the last years, baculoviruses have also been developed as expression vectors for the production of foreign proteins and therapeutic products (Kost and Kemp, 2016; Linn et al., 2010; Luckow et al., 1993; Maeda, 1986; van Oers et al., 2015).

The baculovirus infection cycle starts when a susceptible insect host ingests the virus particles, occlusion bodies (OBs) present on plants or soil. In the insect midgut, the polyhedrin matrix solubilizes and the occlusion derived virions (ODVs; infectious units) and viral-infection enhancins are released. In their way to their primary cellular hosts, the midgut epithelial cells, ODVs encounter a first obstacle, the peritrophic matrix (PM) (Rohrmann, 2019). The PM lines the midgut and, as the first barrier to

baculovirus infection, it can be considered a meaningful target in pest control. Functionally, it constitutes a physical and biochemical barrier that protects the midgut epithelium from abrasive food particles, digestive enzymes and *per os* infectious pathogens (Erlandson et al., 2019). Structurally, it is an acellular chitin and glycoprotein layer with the insect intestinal mucin (IIM) as the major protein in lepidopteran insects (Wang and Granados, 2001).

Chemical agents with chitin-binding properties, such as optical brighteners, bind chitin and inhibit PM formation (Wang and Granados, 2001; Zhu et al., 2007). However, optical brighteners are chemical compounds and their inclusion in green products is not accepted. Enhancins, encoded by several baculoviruses, degrade the mucin (Derksen and Granados, 1988; Wang and Granados, 1997) and chitinases are enzymes that hydrolize chitin. Both of them produce pores in the PM (Brandt et al., 1997; Shapiro et al., 1987). Chitinases have been poorly studied as enhancers of baculovirus infections (Shapiro et al., 1987) while the virus-enhancing activity of enhancins from lepidopteran-infecting granuloviruses (GV, genus Betabaculovirus) has been well-documented (Erlandson et al., 2019, Gallo et al., 1991; Gizjen et al., 1995; Lei et al., 2019). Enhancin genes are prevalent in Betabaculovirus, where they are present in the occlusion body (OB) matrix (Lepore et al., 1996; Erlandson et al., 2019). In contrast, orthologues present in lepidopteran-infecting nucleopolyhedroviruses (NPV, genus Alphabaculovirus), are associated with ODV envelopes and their efficacy as enhancers of NPV infections has come from experiments with recombinant viruses (Lepore et al., 1996; Bischoff and Slavicek, 1997; Li et al., 2003; Slavicek and Popham, 2005).

The aims of this thesis were: (i) to evaluate the synergistic activity of a betabaculovirus enhancin produced in an Autographa californica NPV (AcMNPV) expression system to evaluate the synergistic activity on the infectivity of several baculoviruses, and (ii) to determine the location of the betabaculovirus enhancin within the OBs of AcMNNPV. First, an extensive revision of enhancins along with other PM disrupting agents, such as optical brighteners and chitinases that increase baculovirus and other microbial pathogens infectivity, was performed with the aim of selecting an enhancin for its production as a synergistic product. Second, the

selected agent was expressed by the AcMNPV recombinant bacmid and its location within the OBs and the enhancing effect on baculovirus activity was evaluated.

2. BACULOVIRUS BIOLOGY

The discovery of baculovirus disease dates back to the development of the silk industry in China more than 5,000 years ago (Benz, 1986). Traditionally, one of the main problems in silk cultivation has been the financially devastating losses due to a disease affecting silkworms. In the 19th century, with the development of optical microscopy, it was possible to observe the presence of polyhedron-shaped occlusion bodies (Figure 1) and establish a connection between the presence of polyhedra and silkworm disease (Carstens, 2009). But it was not until 1940, with the development of electron microscopy, that Bergold demonstrated the viral nature of polyhedra, with the observation of rod-shaped virus particles within them (Bergold, 1952). The term *baculovirus* was then coined by Mauro Martignoni. It derives from the Latin word *baculum* (rod) to reflect the shape of virions. Finally, in 1976 the *Baculoviridae* family was registered (Fenner et al., 1976).

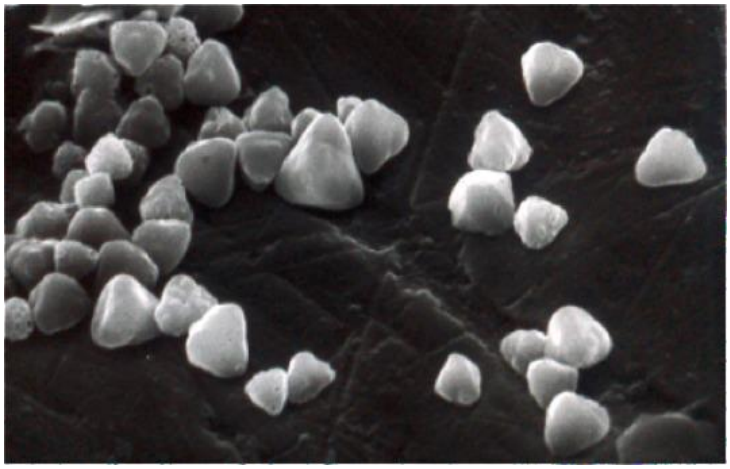

Figure 1. Baculovirus occlusion bodies. Scanning electron microscopy by K. Hughes and R.B. Addison.

2.1. Morphology and taxonomy.

The Baculoviridae is a large family of infectious agents that cause fatal disease in arthropods. They have a double-stranded circular DNA genome packaged in rod-shaped nucleocapsids (Ackermann and Smirnoff, 1983; Federici, 1997). Nucleocapsids, the elementary genetic units of baculoviruses, are enveloped by a lipoprotein membrane acquired from either the host cell plasma membrane to form the budded virions (BVs), or the inner nuclear membrane to form occlusion-derived virions (ODVs) (Blissard and Rohrmann, 1990; Clem and Passarelli, 2013; Rohrman, 2019). The structure of the virion is critical to the ability of baculoviruses to replicate and spread efficiently within insects and then to transmit the infection throughout the population. Although these two types of virions are similar in their nucleocapsid structure, they differ in the origin and composition of their envelopes and their roles in the virus life cycle (Funk et al., 1997; Hou et al., 2013). All baculovirus virions share the same basic structure and constitute the morphological unit responsible for viral infection. BVs form early in infected cells and are responsible for the dissemination of the disease inside the insect body (Rohrmann, 2019). They contain a single nucleocapsid, formed when budding from the host cell plasma membrane at recognition sites containing two glycoproteins, GP64 and F-protein, which become integrated in the virion membrane and allow recognition of new target cells. Based on the type of fusogenic protein, gp64 and F-protein, NPVs were classified in Group I and Group II, respectively (Blissard and Wenz, 1992; Rohrmann, 2019) (Figure 2). ODVs obtain their envelope from the membrane that internally surrounds the cell nucleus, and can enclose one or more nucleocapsids, generating two different phenotypes, single and multiple. ODVs are the infectious units and produced in the very late stage of infection. They become embedded in a protein matrix (polyhedrin or granulin) that crystalizes around one ODV or more ODVs forming a distinct structure known as occlusion body (OB). The OB is responsible for the horizontal transmission of the virus and is adapted to survival in the environment (Rohrmann, 2019). The OBs are highly stable and protect the virions from environmental damage when outside their hosts.

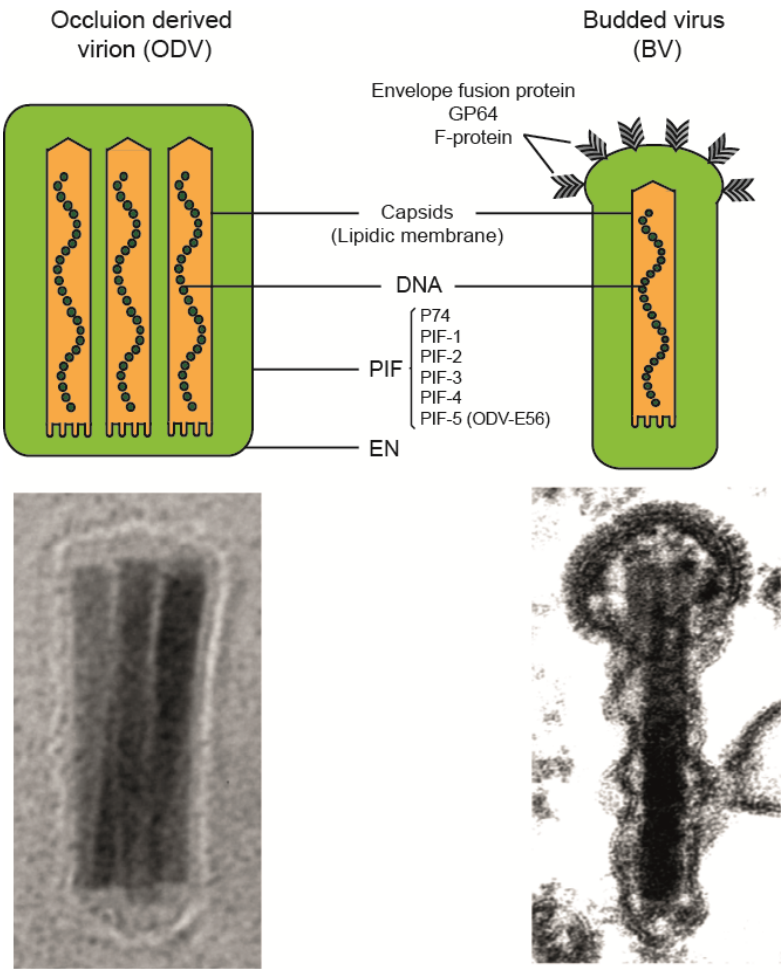

Figure 2. The two virions phenotypes. Above a schematic diagram and below transmission electron microscopy (TEM). Occlusion derived virions (ODV) and budded virus (BV) content identical nucleocapsids, but differ in the protein and lipid composition of their envelopes.

Formerly, the family Baculoviridae was classified into two genera according to the morphology of the OBs: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Murphy et al., 1995). In NPVs, the protein matrix is composed mainly by polyhedrin, the virion sizes range from 0.15 to 15 µm and more than one nucleocapsid can be enveloped within each virion. NPVs with only one nucleocapsid per ODV are termed single nucleopolyheroviruses (SNPV), whereas NPVs containing two or more nucleocapsid per ODV are designated multiple nucleopolyherovirus (MNPV) (Figure 3). In GVs, the granulin is the major component of the OB protein matrix. ODV sizes

vary between 0.13 and 0.50 µm and only one ODV (rarely two or more) is occluded within them (Friesen and Miller, 2001) (Figure 3).

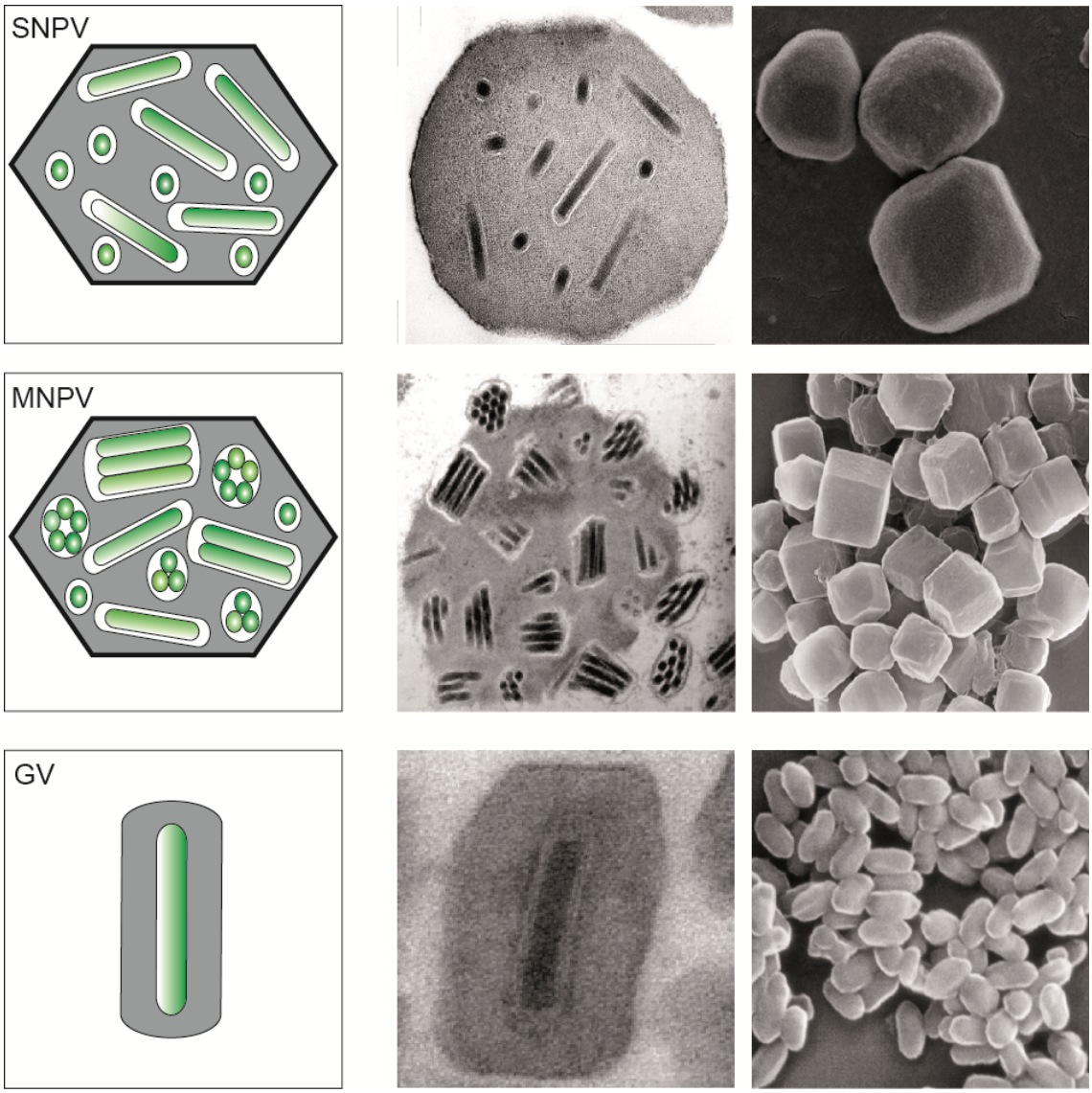

Figure 3. Morphological differences of occlusion bodies (OBs) between single (top row), multiple (middle row) nucleopolyhedroviruses, and granuloviruses (bottom row). The left column shows a schematic representation of OB cross-sections; the middle column shows OB cross-section as observed with a transmission electron microscopy (TEM) and the third column shows OBs as observed by scanning microscopy.

More recently, the International Committee on Taxonomy of Viruses (ICTV) accepted a new classification based on the phylogenetic analysis of 38 genes that

shows that the family contains four monophyletic groups (Fig. 4). These define the current four genera, which can also be separated according to the taxonomical orders of their insect hosts and on their morphology (Harrison et al., 2018; Jehle et al., 2006) and which are: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs), and *Deltabaculovirus* (dipteran-specific NPVs) (Jehle et al., 2006; King et al., 2011). The ICTV has incorporated the Baculoviridae family into the taxonomical order Lefavirales (ICTV, 2020).

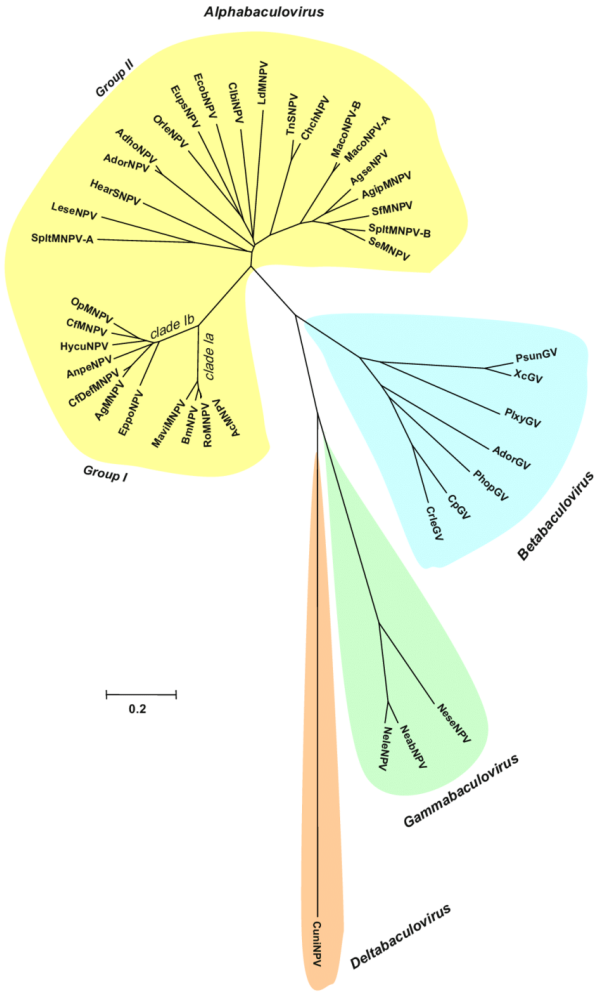

Figure 4. The baculovirus phylogeny. The baculovirus tree consists of four major clades based on the alignment of amino acid positions of sequenced baculoviruses. The best-studied genus is *Alphabaculovirus*, which includes all the lepidopteran-specific nucleopolyhedroviruses. This figure was uploaded from Harrison and Hoover (2012).

2.2. Baculovirus pathogenesis

2.2.1. *In vivo* replication and pathogenesis

The baculovirus infection cycle varies depending on the virus genera (Jehle et al., 2006). While *Alphabaculovirus* and *Betabaculovirus* show a secondary infection and are able to infect all host tissues, the replication of *Deltabaculovirus* and *Gammabaculovirus* is restricted to the midgut, which limits their development as bioinsecticides. The primary infection of Lepidopteran-infecting NPVs and GVs occurs when a susceptible insect larva ingests the OBs present in the environment (soil, foliage, etc.) (Fuxa, 2004) (Figure 5). When they reach the midgut, the ingested OBs are solubilized in the highly alkaline midgut digestive juice, at a pH ranging 9.5 to 11.5 (Jaques, 1985) and ODVs and some infection-enhancing factors are released. The ODVs pass through the peritrophic membrane (PM) aided by viral enhancins and host cell proteinases (Wang and Granados, 1997; Washburn et al., 1995), which degrade the mucin component of the PM.

Once ODVs overcome the PM, they come into contact with their primary cellular target: the midgut epithelial cells. The process of ODV attachment and entry depends on several proteins present on their envelope, the *per os* infectivity factors (PIFs). They form a complex that interacts with an unknown receptor and mediate fusion with midgut epithelial cell membranes (Boogaard et al., 2020; Mu et al., 2014; Wang et al., 2017). The nucleocapsids are then released into the cytoplasm and transported to the nucleus, where viral DNA is uncoated. Then, viral DNA replication and formation of progeny nucleocapsids takes place (Horton and Burand, 1993). Viral proteins of the BV envelope, GP64 or F-protein, which confer tissue specificity for secondary infection, are transported to the cell surface and incorporated into the viral envelope, and newly formed nucleocapsids are initially released by budding through the plasma membrane of the infected cell, forming the BVs (Wang et al., 2014). BVs then pass into the hemocoelic cavity through tracheoles, avoiding the basal membrane (Engelhard et al., 1994; Rohrmann, 2019). They are able to enter cells of other tissues by endocytosis, where they initiate a secondary infection process. Early in the systemic infection, more BVs are produced, to spread the infection throughout the insect. The insect tracheal system and haemolymph are principally responsible

for the successful transport of the BVs throughout susceptible tissues such as fat body, muscles and haemocytes (Flipsen et al., 1996).

Once systemic infection is broadly spread, newly formed nucleocapsids remain in the host cell nucleus and assemble into ODVs. Later in infection, ODVs are wrapped in an envelope, followed by occlusion in polyhedra (OB). At this time, the synthetized enhancins are packaged; in the OBs of betabaculoviruses or in the ODV envelope in alphabaculoviruses (Erlandson et al., 2019). At the end of the systemic infection, the nuclear and the plasma membranes breakdown, liberating the OBs and other cellular contents into the haemocoel. Virus encoded proteins, chitinase and cathepsin (O'Reilly, 1997), intervene in the subsequent degradation of the larval cuticle and hence in the releasing of OBs in the environment (Federici, 1997).

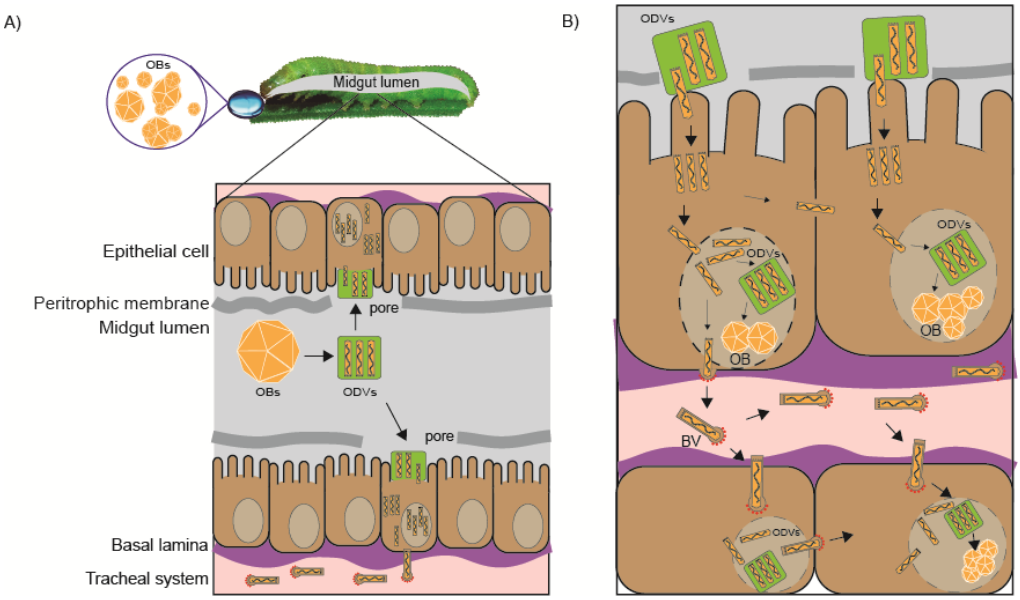

Figure 5. Squematic representation of *Alphabaculovirus* primary (A) and secondary (B) infections.

Typical signs and symptoms of infection can be observed in lepidopteran larvae. Internally, host cell metabolism and RNA synthesis increase, whereas hormonal titers in the larva are also affected (Rohrmann, 2019). The external signs of disease appear some days after infection, usually at the final stages (Granados and Williams, 1986). Some of these include changes in the color and luster of the

integument, loss of appetite and lower activity. Larvae also modify their behaviour in other ways. At the end of the infection, the baculovirus induces enhanced locomotory activity and the larvae climb to the top of the plants (Hoover et al., 2011). Dead larvae hang typically from their last abdominal pseudopods. There is also a process of disintegration and liquefaction, in which the cathepsine and chitinase enzymes play a crucial role to release the OBs in the environment.

2.2.2. *In vitro* replication.

The establishment of insect cell lines in specific insect cell culture media (Gaw et al., 1959; Grace, 1967; Hink, 1970) and the observation that the haemolymph of AcMNPV-infected caterpillars was highly infectious for cultured insect cells (Vaughn and Faulkner, 1963) was crucial for the development of cell culture techniques. These have contributed enormously to unravel the molecular biology of baculoviruses. The replication and morphogenesis of baculoviruses have been studied most intensely in cell culture systems (Blissard and Rohrmann, 1990; Erlandson, 2009; Granados and Lawler, 1981; Hitchman et al., 2007), which have provided fundamental knowledge of baculovirus DNA replication, gene function, gene expression, and gene regulation. Several other baculovirus characteristics, such as host specificity and baculovirus diversity present within natural populations can be investigated also using this system (Blissard and Rohrmann, 1990; Williams et al., 2011). Many insect cells are susceptible to baculovirus infection and it is relatively easy to establish continuous cultures of insect cells *in vitro*. The most used system is that of *S. frugiperda* cell line (Sf21) (Vaughn et al., 1977) with AcMNPV. Several other baculoviruses have been grown in cell culture using different cell lines, including Helicoverpa armigera SNPV in HzAM1, established from ovarian *H. zea* cells (McIntosh et al., 1999) and Trichoplusia ni SNPV in BTI-Tn-5B1-4 (Granados et al., 1994). However, few baculovirus-cell culture systems match the efficiency of the AcMNPV-Sf21 system.

In cell culture, the first step of baculovirus infection, which implies the infection of midgut by dissolution of the OBs, is circumvented. Instead, virus infection may be established most conveniently with BVs obtained from cell culture or from the haemolymph of infected larvae. Alternatively, virus infection can also be established with the ODVs released from an alkaline treatment of OBs. However, continuous

passage of viral progeny in cell culture favours the passage effect, i.e., the formation and accumulation of defective interfering particles (DIPs). These are deleted genotypes lacking genomic regions with genes essential for primary infections (Heldens et al., 1996) or auxiliary genes (Dai et al., 2000: Pijlman et al., 2001, 2002), not necessary for secondary infection.

From a practical point of view, cell-virus systems have proven useful not only for generating engineered baculoviruses for biological control (Moscardi, 1999) or for the expression of novel genes (Jarvis, 1997), but also for the expression of heterologous genes and for the production of valuable proteins for therapeutic purposes (Kost and Kemp, 2016).

2.3. Genome organization and molecular biology of baculoviruses.

Baculoviruses have a circular double stranded genome (Rohrmann, 2019). Currently, there are 90 complete baculovirus genomes deposited in GenBank (NCBI). These include 60 *Alphabaculovirus*, 26 *Betabaculovirus*, 3 *Gammabaculovirus* and 1 *Deltabaculovirus*. *Gammabaculovirus* and *Deltabaculovirus* have shorter genomes, probably because these baculoviruses are only infective to midgut host cells (Rohrmann, 2019; Ishimwe et al., 2015). In contrast, *Alphabaculovirus* and *Betabaculovirus* show more variability in their genome sizes (Jehle et al., 2006).

The genome sizes vary from about 80 to over 180 kb and encode between 90 and 180 genes (Van Oers and Vlak, 2007). Baculovirus open reading frames (ORFs) are tightly packed with minimal intergenic regions and the coding sequences are almost equally distributed over both strands and as non-overlapping contiguous sequences. A set of 38 genes has been identified in all sequenced baculovirus genomes, known as the core genes (Aragão-Silva et al., 2016; Rohrmann, 2019). They represent a hallmark of the virus family, are considered crucial for the life cycle of baculoviruses, and may play important roles in common biological functions (Lapointe et al., 2012) such as: infecting host midgut cells, encoding the viral RNA polymerase, or forming the structure of virion-associated proteins (capsid and ODV envelope). They may also be important for the infectivity of larvae (Miele et al., 2011; Lapointe et al., 2012; Rohrmann, 2019). Auxiliary genes are not essential for viral

replication but provide selective advantage to the virus (Bivian-Hernández et al., 2017; O'Reilly, 1997; Yang et al., 2016). The enhancin and quitinase genes are included within this group (Miller, 1997; O'Reilly, 1997). Finally, genes unique to each virus species, or characteristic of the different baculovirus genera, might influence individual phenotypic traits of particular virus species (Herniou et al., 2003).

Detailed analysis of the regulated cascade of gene expression showed that baculovirus genes can also be classified depending on the time they are expressed into four classes: early, delayed early, late, and the special class of very late genes which comprises the *polh* and *p10* genes (Figure 6) (Passarelli and Guarino, 2007; Rohrmann, 2019; van Oers, 2011; van Oers et al., 2015). Early viral genes contain promoters recognized by host RNA polymerases. Both late and very late genes contain TAAG motifs in their promoters. These genes are transcribed by a virus-encoded RNA polymerase complex consisting on four subunits (van Oers et al., 2015). The Polh and P10 proteins are produced in large amounts at the end of the infection to form, respectively, the OBs and the fibrillary structures in the nucleus and cytoplasm, which are required for efficient OB release from infected cells (van Oers and Vlak, 1997; van Oers et al., 2015). The class of very late genes is unique to baculoviruses. The two very late genes are unnecessary for progeny virus production in cell culture, but indispensable for horizontal transmission. This implies that the very strong *polh* and p10 promoters can be used in the context of a baculovirus infection to drive foreign gene expression (van Oers et al., 2015).

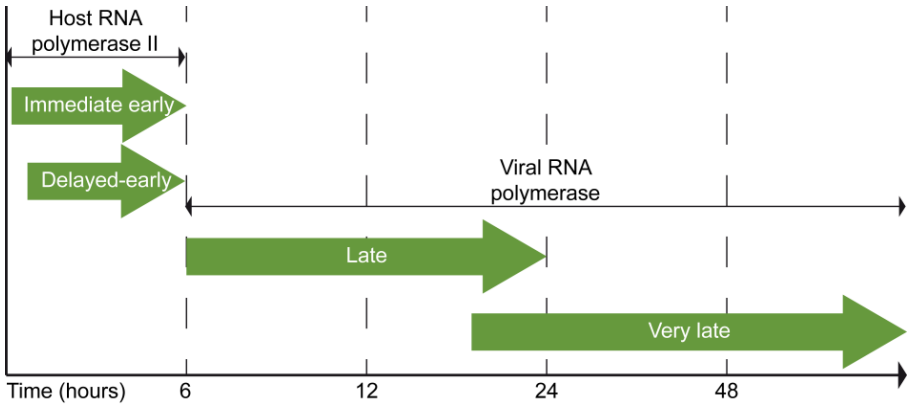

Figure 6. Squematic representation of the four phases of baculovirus gene expression.

3. BACULOVIRUS APPLICATIONS

Baculoviruses are very versatile entities that have allowed the development of whole viruses or some of their individual components into different biotechnological applications. From the time their diseases were acknowledged, 5,000 years ago in China as a great epizootic that damaged the silk industry, baculoviruses have been developed as effective bioinseticides for the control of lepidopteran pests, and their major use dates from the first half of the XX century (Cory and Bishop, 1997). Years later, in the 1980s, the development of biotechnology allowed the development of expression vectors based on these baculoviruses for the production of recombinant proteins and therapeutic treatments (Airenne et al., 2013; Boyce and Bucher, 1996; Hofmann et al., 1995; Linn et al., 2010).

3.1. Baculoviruses as bioinsecticides

Baculoviruses have been recognized as efficient biopesticides for the control of lepidopteran insect species in agriculture and forestry (Black et al., 1997; Inceoglu et al., 2006; Moscardi, 1999; Szewczyk et al., 2006). They are naturally occurring pathogens highly specific to insects, with some viruses infective to only one or two insect species (Moscardi, 1999; Caballero et al., 2009). Consequently, they are safe for vertebrates, humans and non-target organisms, and contribute to maintain the biodiversity in agroecosystems (Ahmad et al., 2011; Ashour et al., 2007; Gröner, 1986). Moreover, their high persistence in the environment allows their efficient horizontal transmission to new susceptible insects(Williams et al., 2017). Furthermore, the fact that they can be applied by conventional methods, favours their commercial development (Moscardi, 1999).

Baculoviruses have showed high efficacy in controlling field pests, and around 60 baculovirus-based pesticides have been developed globally (Figure 7). The most prominent example is the Brazilian program against *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), which has successfully controled outbreaks of this pest since 1982 in more than 2 million soybean hectares with a local A. gemmatalis multiple nucleopolyhedrovirus (AgMNPV) (Moscardi, 1999; Sosa-Gómez et al., 2008; Szewczyk et al., 2006; Yang et al., 2012). In Europe, Cydia pomonella granulovirus (CpGV) has been used to protect pome fruit crops from the key pest *C. pomonella* L.

(Lepidoptera: Tortricidae) (Kutinkova et al., 2012; Lacey et al., 2008; Moscardi, 1999; Vincent et al., 2007). A more familiar example is that occurring in greenhouses in southern Spain, where *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) populations resistant to chemical insecticides have been controlled efficiently with SeMNPV (Caballero et al., 2009). Furthermore, the gypsy moth, *Lymantria dispar L.* (Lepidoptera: Erebidae), an important defoliator in forestry, is being controled worldwide with its homologous nucleopolyhedrovirus, LdMNPV (Moscardi et al., 2011). The majority of baculoviruses developed as biopesticides are nucleopolyhedroviruses (NPV; alphabaculoviruses) and only a few are granuloviruses (GV; betabaculoviruses) (Caballero et al., 2009; Inceoglu et al., 2006).

Figure 7. Some of the currently commercialized baculovirus-based products, such as Coopervirus based on Anticarsia gemmatalis NPV to control *Anticarsia gemmatalis* damage in soybean crops in Brazil, Gemstar based on Helicoverpa zea SNPV against *Heliothis* and Helicoverpa spp. to protect maize, tomato, cotton and tobacco or VIR-EX based on Spodoptera exigua NPV to treat greenhouses crops in Spain.

Unfortunately, baculovirus-based bioinsecticides present several limitations when compared with chemical insecticides (Ignoffo et al., 1977). Their speed of kill is lower, and it takes them several days to kill the larvae (Caballero et al., 1992; Shapiro, 1986). During this period, larvae continue feeding and causing crop damage. The improvement of the virulence has been mainly achieved by the construction of recombinant baculoviruses either by expressing insecticidal toxins, enzymes or hormones, or by deleting genes involved in the host metabolism, such as *egt* (Bonning et al., 1995; Hammock et al., 1990; Maeda, 1986; O'Reilly and Miller, 1991; Stewart et al., 1991; Tomalski and Miller, 1991). However, only a few genetically modified baculoviruses have been tested in the field (Eberle et al., 2012) and none

have been commercialized (Summers, 2006) owing to public aversion to genetically modified organisms (GMOs) (Szewczyk et al., 2006). In some instances, addition of synergistic factors such as optical brighteners or enhancins have reduced the time required to kill the larvae (Gallo et al., 1991; Shapiro and Argauer, 2001).

Another major drawback of baculoviruses is their high susceptibility to UV degradation. For this reason, appropriate formulations with UV protectants have been the subject of thorough research (Copping and Menn, 2000). Optical brighteners have been extensively assayed under laboratory and field conditions as UV protectants (Dougherty et al., 1996; Zhu et al., 2013).

The narrow host range of baculoviruses, an advantage from an environmental standpoint, is at the same time undesirable at the commercial level for crops in which two or more insect pests are present. The mass production is one of the greatest limitations to the use of baculoviruses as biocontrol agents (Grzywacz et al., 1998; Gupta et al., 2007; Hunter-Fujita, 1998). As obligate intracellular pathogens, baculoviruses need active host cells for their replication, therefore, viral production must be performed in susceptible insect cells. To date, the only feasible method for the production of baculovirus-based insecticides has been *in vivo*, using insects (Gupta et al., 2007; Hunter-Fujita, 1998). *In vivo* production involves the inoculation of massive numbers of larvae, their rearing throughout virus infection, and the recollection and purification of OBs from larval cadavers. This process is costly and makes it difficult for viral products to compete with chemicals in the marketplace (Grzywacz et al., 1998). Incorporation of synergistic compounds such as optical brighteners, enhancins or chitinases to baculovirus formulations have shown to reduce lethal doses (Bernal et al., 2014; Hayakawa et al., 2000; Lasa et al., 2009, Shapiro and Robertson, 1992; Wang et al., 1994).

3.2. Baculoviruses as expression systems

In 1982 *E. coli* produced human insulin, the world's first recombinant DNA drug, was approved by the FDA (Kost and Kemp, 1987). Since this historical event, remarkable progress has been made in developing bacterial, yeast, mammalian and insect cell protein expression systems that are used to produce recombinant proteins

for both research and clinical applications. Of the available approaches, the insect cell based baculovirus expression vector system (BEVS) has proven to be a particularly adaptable system for producing a diverse collection of proteins.

In 1983, Summers and colleagues reported the expression of human IFN-β with a recombinant AcMNPV by exploiting the polyhedrin promoter (Smith et al., 1983). At the same time, Miller and co-workers were able to express the *Escherechia coli* β-galactosidase using the same approach (Pennock et al., 1984). During the last years, baculovirus based expression technology has matured and is now commonly used to produce proteins of scientific interest as well as commercial vaccines worldwide, both for human and veterinary use (van Oers et al., 2015). BEVS have been used for production of the human papilloma virus vaccine, Cervarix, the first FDA approved insect cell produced product (Deschuyteneer et al., 2010), and FluBlok, a vaccine based on the influenza virus hemagglutinin protein (van Oers et al., 2015). Modified baculoviruses containing mammalian promoters (BacMam viruses), have proven to be efficient gene delivery vectors for mammalian cells and provide an alternative transient mammalian cell based protein expression approach to that of plasmid DNA based transfection methodologies (Ames et al., 2009). The baculovirus-expression systems are also promising systems to produce viral vectors for gene theraphy, mainly adeno-associated virus (AAV) vectors, using co-infection of baculovirus vectors and lentiviral vectors (Lesch et al., 2008; Urabe et al., 2002).

Typical properties of baculoviruses, such as the high level of very late gene expression and the fact that polyhedrin and p10 genes are not necessary for viral replication in cell culture, make them highly suitable as vectors for foreign expression. As a consequence, the polh and p10 promoters can be used to drive the expression of foreign genes in insect cells (van Oers, 2011; van Oers et al., 2015). In principle, any foreign gene may be expressed in the baculovirus-insect cell expression system, and often biologically active and/or immunogenic proteins are produced. An additional advantage is that baculoviruses have limited host range (insects) and are hence safe for vertebrates (Kost and Kemp, 2016). Baculovirus vectors are used in combination with insect cells or larvae to produce high levels of recombinant proteins and provide appropriate postranslational modifications in contrast to prokaryotic expression systems (van Oers, 2011; van Oers et al., 2015). Two baculovirus species are commonly used for foreign gene expression, the most used ones are AcMNPV,

the type species of the genus *Alphabaculovirus*, and BmNPV, another *Alphabaculovirus* that replicates in silkworm. For protein production with AcMNPV vectors, insect cell cultures are used (up to 1000 L scale) while BmNPV vectors can be used in cell culture or silkworm larvae (van Oers, 2011; van Oers et al., 2015).

The classical way of making recombinant viruses is by homologous recombination between the viral genome and a transfer plasmid carrying the foreign gene under control of the p10 and polh promoter (Smith et al., 1983; Vlak et al., 1990). Plasmids with multiple promoters have also been developed for classical recombination systems (Weyer and Possee, 1991), although with lower recombination rates (<0.01%). Then, recombinant viruses need to be purified following a procedure that requires several rounds of plaque purification using an agar overlay to isolate recombinant from parental, wild-type virus (King and Possee, 1992; O'Reilly et al., 1994). As this purification is quite a time-consuming process, the original method was improved using baculovirus genomes linearized at the desired insertion site (Kitts et al., 1990; Martens et al., 1995), enabling a higher recovery rate (of ≈30%) of recombinants after recombination with the transfer plasmid (Kitts and Possee, 1993). This method uses the BacPAK6 vector (Kitts and Possee, 1993) or the commercially available Baculogold (BD Biosciences) and BacN blue vectors (Invitrogen).

A major step forward was the construction of bacterial artificial chromosomes containing the genome of the baculovirus AcMNPV, as so called bacmids, which allow the manipulation of the viral genome in *E. coli* (DH10ß-strain) (Luckow et al., 1993) (Figure 8). Foreign genes are introduced into the bacmid by site-directed transposition in *E. coli* carrying the AcMNPV bacmid (DH10BAC cells), using Tn7-recombinase mediated transposition. Once the recombinant bacmid is generated and the presence of the transgene and absence of the parental bacmid verified, by PCR for example, the bacmid DNA is used to transfect insect cells; in the case of AcMNPV these cells are typically *Spodoptera frugiperda* Sf9 or *Trichoplusia ni* Tn-5B1-4 (High Five) cells (Vaughn et al., 1977; Wickham et al., 1992). Once inside the cells, the bacmid DNA is transcribed to allow early gene expression, and the expression cascade is initiated. BV particles can be harvested from the culture supernatant and used to infect new cells to produce high titer seed stocks to allow recombinant protein production. This is the methodology used by the Bac-to-Bac® Baculovirus

Expression System (ThermoFisher). Various commercial transfer vectors are available for the bacmid system to allow expression of one or two proteins (pFastBac1 and pFastBacDual; ThermoFisher) and to automatically add tags or signal peptides to the expressed proteins. The Bac-to-Bac® provides a rapid and efficient method to generate recombinant baculoviruses (Figure 8) (Ciccarone et al., 1997). Further improvements came from the vectors that use TOPO cloning or GATEWAY technology (ThermoFisher) to insert the foreign gene into the transfer vector. The BVBoost system makes use of the negative-selection marker sacB (Airenne et al., 2003) or the BacMagic (Merck) and BaculoOne (PAA) systems allow for high-throughput expression of foreign genes, e.g. for the expression of a cDNA library using semi-automated methods for generating recombinant viruses (flashBac; Oxford Expression Technologies Ltd.) (Possee et al., 2008).

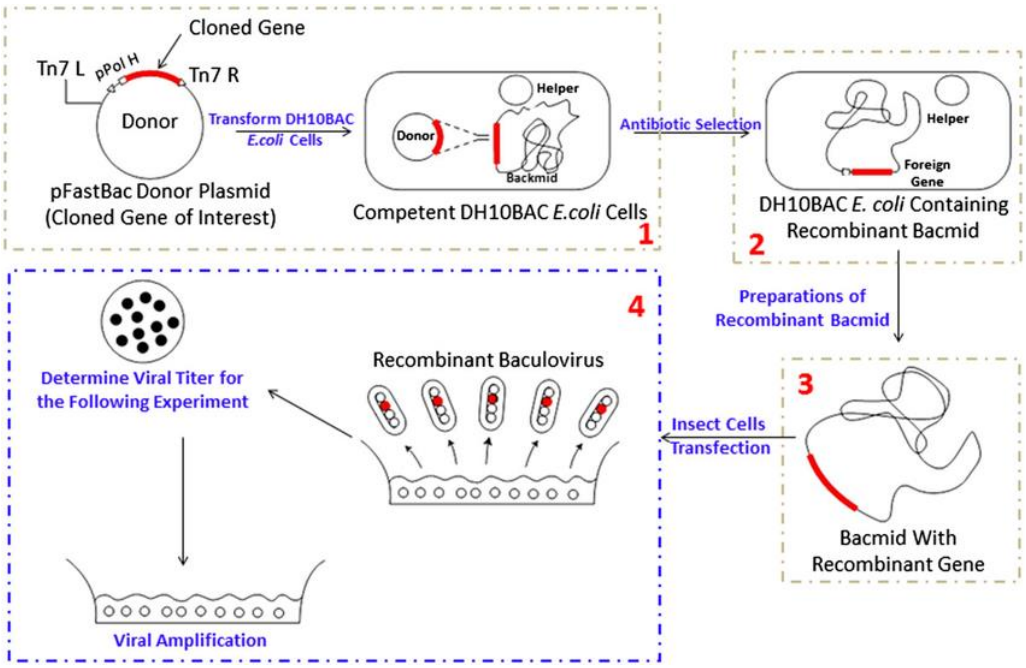

Figure 8. Recombinant baculoviruses and gene expression protocol using the bac-to-bac expression system; step 1. Construction of donor plasmid; step 2. Production of bacmid; step 3. Recombinant bacmid preparation; step 4. Production of recombinant baculovirus. (Sui et al., 2014).

4. THE PERITROPHIC MATRIX AS A TARGET SITE FOR INSECT CONTROL

4.1. Structure of the peritrophic matrix

The first barrier that baculoviruses must overcome to produce a successful infection in susceptible host is the peritrophic matrix (PM). The PM lines the insect midgut and represents a major lepidopteran physical barrier against microbial pathogens (Erlandson et al., 2019). Its main role is to protect midgut epithelial cells from food particles, digestive enzymes, and pathogens (Erlandson et al., 2019; Wang and Granados, 2001). To accomplish this function, the PM is primarily composed of chitin and proteins, which include glycoproteins and proteoglycans, being the insect intestinal mucin (IIM) the major PM protein (Wang and Granados, 2001).

In lepidopteran larvae, the binding of PM structural proteins to PM chitin appears to be the mechanism for PM formation (Wang and Granados, 2001). PM structural proteins are mainly associated with the chitin fibrils by high-affinity non-covalent binding via chitin-binding domains. The binding of PM structural proteins to PM chitin fibrils appears to be a major mechanism for Type 1 PM formation in lepidopteran larvae (Wang and Granados, 2001). The IIM, as well as other PM chitin-binding proteins, cross-links the chitin fibrils through its multiple chitin-binding domains, leading to a mucinous structure. Meanwhile, the mucin domains from the IIM are highly resistant to protease degradation and likely shield other PM components from digestive enzymes (Brandt et al., 1978; Erlandson et al., 2019; Wang and Granados, 1997). Furthermore, the strong binding of PM proteins to chitin may also protect both the proteins and the chitin from degradation by midgut enzymes, either endogenous or acquired from food materials. The chitin-binding domains are rich in cysteine and their formation of disulfide bonds contributes to the stability of the chitin-binding function. Incorporation of chitin fibrils significantly increases the physical strength of a PM with a limited thickness (Wang and Granados, 2001).

4.2. PM disrupting agents

The important physiological functions of the PM make it a significant structural target for insect control. Disruption of the link between chitin and proteins in the PM

structure increases PM permeability and leads to the collapse of the midgut defence against pathogens. For instance, in a baculovirus infection, such a disruption favours virus passage to midgut cells and, consequently, reduces the virus concentration needed to produce a successful infection. For this reason, the PM represents an excellent target for pest biocontrol (Figure 6) (Wang and Granados, 2001).

Inhibitors of chitin synthesis can be potential insect control agents (Wang and Granados, 2001). Chemical agents with chitin-binding properties, such as optical brighteners, bind chitin and inhibit PM formation through the formation of pores in the PM. This facilitates virus passage to midgut cells (García-González and Genersch, 2013; Wang and Granados, 2001; Zhu et al., 2007), increasing susceptibility to microbial pathogens. Optical brighteners produced by far the highest enhancement of baculovirus infections. However, optical brighteners are catalogued as chemical compounds and their inclusion as adjuvants in green products (eg. baculovirus formulations), is not accepted. Nonetheless, other compounds that perform these functions and meet environmentally friendly standards are being found in nature. Some insect baculoviruses have evolved accessory gene products, such as enhancins, that degrade the PM and therefore, enhance initial virus infection of the midgut cells (Erlandson, et al., 2019; Slavicek, 2012; Wang and Granados, 2001). Chitinases are enzymes that breakdown chitin and have been detected in a great variety of organisms (containing or non-containing chitin) as defensive systems. Some of them have been shown to affect the PM structure (Berini et al., 2016; Rao et al., 2004).

Enhancins have been extensively studied as enhancers of baculovirus infections (Biedma et al., 2015; Gallo et al., 1991; Gizjen et al., 1995; Goto, 1990; Guo et al., 2007; Jeyarani and Karuppuchamy, 2010; Lei et al., 2019; Tanada et al., 1975). In contrast, only one study reported an enhancement of baculvoirus infection by adding purified chitinases (Shapiro et al., 1987). The effect of recombinant chitinases expressed on viruses or plants has been investigated more deeply (Corrado et al., 2008; Durechova et al., 2019; Fiandra et al., 2010; Gopalakrishnan et al., 1995; Wang et al., 2013).

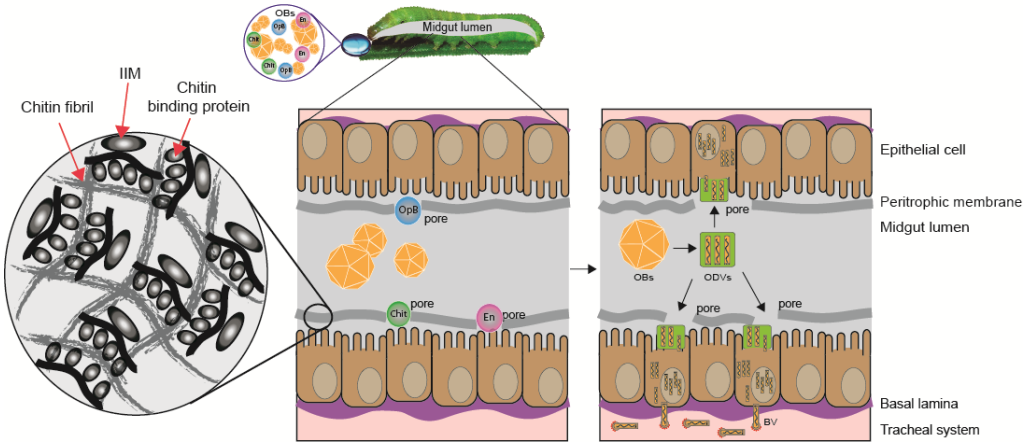

Figure 9. Schematic representation of synergistic substances that exert a disruptive effect on the peritrophic membrane.

4.2.1. *Enhancins*

Enhancin refers to a synergistic or viral factor, found firstly in GVs and later in a few NPVs, that has the ability to enhance the infection of other NPVs (Biedma et al., 2015; Derksen and Granados, 1988; Gallo et al., 1991; Gizjen et al., 1995; Guo et al., 2007; Jeyarani and Karuppuchamy, 2010; Lei et al., 2019; Tanada et al., 1971, 1973). Enhancin genes have been identified in approximately 30% of NPVs and GVs sequenced to date. These genes are more common in GVs, present in about 46% of the genomes (Bivian-Fernández et al., 2012; Hayakawa et al., 1999; Li et al., 2021). In contrast, enhancin orthologues are found in a few alphabaculoviruses (Bischoff and Slavicek, 1997; Jakubowska et al., 2006; de Jong et al., 2005; Li et al., 2002a 2002b, 2003; Popham et al., 2001). Through genome sequencing, enhancin genes have also been identified in the genomes of several bacteria and fungi (Ivanova et al., 2003; Parkhill et al., 2001; Read et al., 2003). However, their role as enhancers of infectivity has been poorly investigated.

Baculovirus enhancins act as proteases and degrade the major PM protein, IIM, resulting in the disruption of this structure and increasing access of entomopathogens (Derksen and Granados, 1988; Toprak et al., 2012; Wang et al., 1997). *Enhancin* genes are more prevalent in lepidopteran-infecting GVs, where they are present in the occlusion body (OB) matrix (Erlandson et al., 2019; Roelvink et al.,

1995). In contrast, orthologues from lepidopteran-infecting NPV are present only in a few NPVs in association with the ODVs (Slavicek and Popham, 2005; Toprak et al., 2012). The fact that alphabaculovirus enhancin is localized to ODVs and betabaculovirus enhancin to OB protein fractions make their impact on the PM different. Alphabaculovirus enhancin displays its activity at the site of interaction of ODVs with the PM, in contrast to the more widespread degradation caused by the release of betabaculovirus enhancin after solubilization of the OBs in the midgut. Therefore, degradation of the target IIMs by alphabaculovirus enhancin is more subtle, than the prominent degradation caused by betabaculovirus enhancins (Slavicek, 2012). This has conditioned the synergistic studies carried out by both. The potentiation activity of betabaculovirus enhancins has been extensively studied. In contrast, the location of NPV enhancins within the ODVs hinders their purification and has restricted their study as synergists of viral infection.

5. AIMS OF THE THESIS

This thesis aims to solve one of the greatest limitations for the use of baculoviruses as biocontrol agents: their relatively high cost associated with the mass production system in larvae. In the present thesis, an enhancing factor was expressed and produced using a baculovirus expression system for its use as a synergistic factor in baculovirus-based formulated products.

In **Chapter II**, a review on the literature covering compounds that degrade the PM such as optical brighteners, viral and bacteria enhancins, and chitinases has been performed. A special focus was posed on enhancins that have been largely assayed in combination with baculoviruses. The objective of the chapter was to select a suitable enhancing factor for its expression in a recombinant virus.

In **Chapter III**, a polyhedrin-positive recombinant AcMNPV expressing a betabaculovirus enhancin was constructed. One question concerning the recombinant enhancin expressed by an NPV is whether the recombinant enzyme is occluded within OBs, as in GVs, or integrated into the ODV envelope, as occurs in NPVs. The main objective of the present chapter was to determine whether a betabaculovirus enhancin was incorporated within OBs or enveloped in the ODVs. The localization of the recombinant protein was analyzed by SDS-PAGE and the insecticidal activity of the recombinant OBs was compared with that of wild-type OBs.

Finally, In **Chapter IV**, the above mentioned recombinant En3 was mass produced using the baculovirus expression vector both *in vitro* and *in vivo*. The goal was to evaluate the feasibility of its mass-production with the aim of incorporating the solubilized enhancin as a synergist. Different parameters were studied in both systems such as harvesting time and insect species. The viability of both systems was evaluated. The massively produced En3 was used in combination with different baculovirus species in homologous and heterologous hosts to evaluate its enhancing capacity.

Enhancins are promising additives that reduce the amount of active compound making baculovirus formulations commercially more competitive.

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CHAPTER II

Peritrophic membrane disrupting agents as synergists of baculovirus infections

ABSTRACT

The peritrophic membrane (PM) is the first barrier that baculoviruses may overcome to establish a successful infection. It is composed of mucin and chitin microfibrils linked to glycoproteins and proteoglycans and its disruption leads to increased susceptibility to microbial pathogens. It thus represents a significant structural target for insect control. Several compounds have been found useful as PM disruptors in the last decades: (1) chemical agents with chitin-binding properties, such as optical brighteners, attach to chitin and inhibit PM formation; (2) enhancins, encoded by several baculoviruses, degrade the mucin component; (3) chitinases, enzymes that hydrolize chitin, produce pores in the PM. This short review aims to gather studies on the synergistic effect of optical brighteners, enhancins and chitinases as PM disrupting agents. We discuss their potential use as synergic agents in formulated baculovirus products.

1. INTRODUCTION

The Baculoviridae is a large family of virus causing fatal disease in arthropods. They have been isolated from more than 700 insect species, most of them (> 80%) belonging to the order Lepidoptera (Herniou et al., 2003; Harrison et al., 2018; Jehle et al., 2006). These viruses are important mortality agents that naturally regulate lepidopteran populations (Ahmad et al., 2011; Clem and Passarelli, 2013; Moscardi, 1999; Rodgers, 1993) and have been recognized as efficient biopesticides for the control of lepidopteran pest species in agriculture and forestry (Black et al., 1997; Inceoglu et al., 2006; Moscardi, 1999; Szewczyk et al., 2006).

The baculovirus infection cycle starts when a susceptible insect ingests the occlusion bodies (OBs) present on plants or in the soil. In the midgut, the polyhedrin matrix solubilizes and the occlusion derived virus (ODVs; infectious units) and virus-structural enhancins are released. Then, ODVs must pass the peritrophic matrix (PM) to reach the midgut cells (Rohrmann, 2019). The PM, a sheet composed by mucin and chitin microfibrils linked to glycoproteins and proteoglycans (Dias et al., 2019; Erlandson et al., 2019; Peters, 1992; Lehane, 1997), is a physical and biochemical barrier. It protects the midgut epithelium from abrasive food particles, digestive enzymes and *per os* infectious pathogens (Erlandson et al., 2019; Hegedus et al., 2009; Terra, 2001; Wang and Granados, 2001).

Lepidopteran larvae typically have a type I PM (Merzendorfer et al., 2016; Shi et al., 2004; Wang and Granados, 2001) with a number of different proteins varying from two to about 30 with molecular weights ranging from 14 to over 200 kDa (Merzendorfer et al., 2016; Wang and Granados, 2001). Some of them are secreted intestinal mucins with chitin-binding affinity. The insect intestinal mucin (IIM) is the major protein in type I PM (Shi et al., 2004). To date, all identified and characterized PM proteins comprise (Dias et al., 2019; Shi et al., 2004) multiple chitin-binding domains to allow the assembly of chitin fibrils and proteins into a mucinosus structure supported by the former (Peters, 1992; Shi et al., 2004; Wang and Granados, 1998, 2001). Glycoproteins, for their part, serve as binding sites for a variety of pathogens, limiting their access to midgut epithelium cells (Erlandson et al., 2019; Wang and Graandos, 1998).

Dissociation of PM proteins from chitin quickly results in degradation of these proteins by endogenous digestive proteases from the gut. In turn, PM disruption leads to increased susceptibility to microbial pathogens (Derksen and Granados, 1998). Hence, the PM can be a significant structural target for insect control. In fact, there are numerous target sites in this protective structure (Wang and Granados, 2001). Chemical agents with chitin-binding properties, such as optical brighteners, bind chitin and inhibit PM formation (García-González and Genersch, 2013; Wang and Granados, 2001). Enhancins, structural proteases encoded by several baculoviruses, specifically degrade the IIM proteins (Derksen and Granados, 1988; Gallo et al., 1991; Tanada et al., 1980; Wang and Granados, 1997). Finally, chitinases, found in a great variety of organisms (containing and non-containing chitin) degrade chitin (Peters, 1992).

In the current review, we focus on evidence confirming the influence of several chemicals and enzymes as PM disrupting agents. The review is set out in sections outlining the sinergystic effect s of optical brighteners, enhancins and chitinases over the infectivity of baculoviruses and other pathogens in lepidopteran larvae. Inclusion of these subtances in OB formulations might increase the susceptibility of lepidopteran larvae to baculoviruses infections, and render infectious particles highly pathogenic to insect pests at the lowest possible number of OBs/larva.

2. OPTICAL BRIGHTENERS

Optical brighteners are used to mask the inherent yellowness of plastics and to improve colors and make plastic look whiter and brighter. Optical brighteners are also known as fluorescent whitening agents. When exposed to UV light these additives absorb the UV light spectrum and reflect it in the visible blue spectrum (Shrivastava, 2018). The latter favored their initial use as UV protectants of baculovirus formulations (Martignoni and Iwai, 1985). Later, Shapiro and Robertson (1992) reported the synergistic effect of optical brighteners on the insecticidal characteristics of the Lymantria dispar nucleopolyhedrovirus (LdMNPV) (Lefavirales: Baculoviridae). Thereafter, several optical brighteners have been tested as activity enhancers of different baculoviruses under laboratory (Dougherty et al., 1996; Hamm, 1999; Hamm and Shapiro, 1992; Shapiro and Dougherty, 1994) and field

(Hamm et al., 1994; Thorpe et al., 1999; Webb et al., 1994, 1994b) conditions (Table 1).

Optical brighteners belong to several chemical groups (e.g., stilbene, oxalone, pyrazoline lactone, coumarin), but stilbene derivatives, such as Blancophor, Leucophor and Tinopal, generated great interest as they showed the highest potentiation rates for baculovirus infections (Table 1) (Hamm, 1999; Lasa et al., 2009; Martínez et al., 2003). The highest enhancement was reported for Leucophor AP at 1%. This stilbene derivate increased the infectivity of Spodoptera exigua NPV (SeMNPV) and the S. frugiperda NPV (SfMNPV) in their homologous fourth intars 5,371 and 2,640 times, repectively (Lasa et al., 2009). Among the stilbene derivates, Tinopal has been the most assayed and showed variable potentiation. Tinopal UNPA-GX at 1% reduced the LC_{50} of Spodoptera litura NPV (SpltNPV) against its homologous L_4 instar by 2,188-fold (Okuno et al., 2003). Tinopal LPW at 1% (wt/vol) potentiated the LdMNPV LC_{50} by 1,670-fold in second instar *L. dispar* (Shapiro and Robertson, 1992), and by only two-fold in Galeria melonella NPV (GmMNPV) on its homologous second instar host (Shapiro and Vaughn, 1995). Surprisingly, an optical brightener not derived from stilbene acid, Phorwhite RKW, increased the LdMNPV infectivity against second instar *L. dispar* to values similar to those reported by stilbene derivates (Shapiro and Robertson, 1992).

Several studies have also reported also an improvement of the LT_{50} (Table 1) (Hamm and Shapiro, 1992; Li and Otvos, 1999; Morales et al., 2001; Shapiro and Argauer, 2001; Vail et al., 1996). What is more, there is also a report on the influence of these chemicals on the host range of a baculovirus and an entomopoxvirus (Shapiro and Dougherty, 1994). Phorwite AR not only reduced the LC_{50} and LT_{50} of LdMNPV on their homologous host but also made *L. dispar* susceptible to Autographa californirca NPV (AcMNPV) and *Amsacta* entomopoxvirus, previously reported as non-infective viruses. Broading the host

Table 1. Synergistic effects of optical brighteners on the insecticidal activity of baculoviruses.

Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Blankophor BA	SfMNPV	*S. frugiperda*	L_3	0.1%	1.37-fold	Droplet	-	Martínez et al., 2003
			L_4	0.1%	23.8-fold	Surface	-	Lasa et al., 2009
			L_4	1%	2738-fold	Surface	-	Lasa et al., 2009
Blankophor BBH	AgMNPV	*A.gemmatalis*	L_4	0.05%	31.1-fold	Diet	-	Morales et al., 2001
	CfMNPV	*C. occidentalis*	L_4	1%	2.7-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	SeMNPV	*S. exigua*	L_2	1%	131-fold	Surface	LT_{50} reduction 0.7-fold	Shapiro and Argauer, 2001
	SfMNPV	*S. frugiperda*	L_3	0.1%	1.5-fold	Droplet	-	Martínez et al., 2003
	LdMNPV	*L.dispar*	L_2	0.3%	213-314-fold	Droplet	-	Thorpe et al., 1999
			-	0.5%	None	Field	Reduction in defoliation and egg-mass density	Thorpe et al., 1999
	LdMNPV	*L.dispar*	L_3	1%	1.5-fold	Field	-	Webb et al., 1994
			L_4	1%	1.5-fold	Field	-	Webb et al., 1994
Blankophor BSU	SeMNPV	*S. exigua*	L_2	1%	1.42- fold	Droplet	LT_{50} Increased by 20%	Shapiro and Argauer, 2001
Blankophor CLE ($C_{30}H_{20}N_6Na_2O_6S_2$)	ChchNPV	*C. chalcites*	L_2	1%	None	Droplet	-	Bernal et al., 2014
			L_4	1%	1.72-fold	Droplet	-	Bernal et al., 2014
	SfMNPV	*S. frugiperda*	L_3	0.1%	None	Droplet	-	Martínez et al., 2003
Blankophor DML	CfMNPV	*C. occidentalis*	L_4	1%	0.7-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	SeMNPV	*S. exigua*	L_2	1%	0.86-fold	Durface	LT_{50} Increased 20%	Shapiro and Argauer, 2001

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Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Blankophor HRS	AgMNPV	*A. gemmatalis*	L_4	0.05%	5.2-fold	Diet	-	Morales et al., 2001
	SeMNPV	*S. exigua*	L_2	1%	374.29-fold	Surface	LT_{50} reduction 0.92-fold	Shapiro and Argauer, 2001
Blankophor LPG	CfMNPV	*C. occidentalis*	L_4	1%	0.74-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	SeMNPV	*S. exigua*	L_2	1%	0.74-fold	Surface	LT_{50} Increased 20%	Shapiro and Argauer, 2001
Blankophor P167	CfMNPV	*C. occidentalis*	L_4	1%	3.1-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	SeMNPV	*S. exigua*	L_2	1%	10.48-fold	Surface	LT_{50} reduction 0.9-fold	Shapiro and Argauer, 2001
Blankophor RKH ($C_{34}H_{32}N_{12}O_6S_2$)	AgMNPV	*A. gemmatalis*	L_4	0.05%	76.6-fold	Diet	LT_{50} decreased	Morales et al., 2001
	CfMNPV	*C. occidentalis*	L_4	1%	1.7-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	LdMNPV	*L. dispar*	L_2	1%	1837.80-fold	Surface	-	Shapiro and Robertson, 1992
	SeMNPV	*S. exigua*	L_2	1%	52.4-fold	Surface	LT_{50} reduction 0.7-fold	Shapiro and Argauer, 2001
Calcofluor M2R	SfaMNPV	*S. exigua*	L_3	1%	1.3-fold	-	Slows growth of larvae	Zhu et al., 2007
	AcMNPV	*T. ni*	L_5	1%	1.6-fold	Diet	Disruption of PM	Wang and Granados, 2000
	AfMNPV	*T.ni*	Neonate	1%	7.8-fold	Surface	LT_{50} decreased	Vail et al., 1996
		Heliothis virescens	Neonate	1%	4.3-fold	Surface	LT_{50} decreased	Vail et al., 1996
		H. zea	Neonate	1%	2.9-fold	Surface	LT_{50} decreased	Vail et al., 1996
		S. exigua	Neonate	1%	13.6-fold	Surface	LT_{50} decreased	Vail et al., 1996
	ChinNPV	*C. includens*	Neonate	0.1%	14.8-fold	Surface	LT_{50} decreased	Zou and Young, 1996
			L_2	0.1%	1583.9-fold	Surface	LT_{50} decreased	Zou and Young, 1996
			L_3	0.1%	1.7-fold	Field	-	Zou and Young, 1996

Table 1. Synergistic effects of optical brighteners on the insecticidal activity of baculoviruses.

Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Calcofluor M2R	ChinNPV	*C. includens*	L_3	1%	2.3-fold	Field	-	Zou and Young, 1996
			L_4	0.1%	1.3-fold	Field	-	Zou and Young, 1996
	SfaMNPV	*S. exigua*	L_4	1%	1.7-fold	Field	-	Zou and Young, 1996
	SfaMNPV	*S. exigua*	L_3	0.1%	1.27-fold	-	Retarded development	Zhu et al., 2007
	SfMNPV	*S. frugiperda*	L_2	0.1%	2.7-fold	Droplet	-	Martínez et al., 2003
		S. frugiperda	L_3	0.1%	6.5-fold	Droplet	-	Martínez et al., 2003
		S. frugiperda	L_4	0.1%	61.6-fold	Droplet	-	Martínez et al., 2003
Leucophor BSB	AgMNPV	*A.gemmatalis*	L_4	0.5%	1.4-fold	Diet	-	Morales et al., 2001
	CfMNPV	*C. occidentalis*	L_4	1%	2-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	LdMNPV	*L. dispar*	L_2	1%	417.70-fold	Surface	-	Shapiro and Robertson, 1992
Leucophor SAC ($C_{48}H_{42}O_{24}S_6$)	ChchNPV	*C. chalcites*	L_2	1%	None	Droplet	-	Bernal et al., 2014
			L_4	1%	2.02-fold	Droplet	-	Bernal et al., 2014
Leucophor SAC	SfMNPV	*S. frugiperda*	L_3	0.1%	None	Droplet	-	Martínez et al., 2003
Leucophor UO ($C_{48}H_{42}O_{24}S_4$)	ChchNPV	*C. chalcites*	L_2	0.1%	0.34-fold	Droplet	-	Bernal et al., 2014
			L_2	1%	1.46-fold	Droplet	-	Bernal et al., 2014
			L_3	0.1%	2.17-fold	Droplet	-	Bernal et al., 2014
			L_3	1%	5.96-fold	Droplet	-	Bernal et al., 2014
			L_4	0.1%	3.87-fold	Droplet	-	Bernal et al., 2014
			L_4	1%	20.08-fold	Droplet	-	Bernal et al., 2014

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Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Leucophor UO ($C_{48}H_{42}O_{24}S_4$)	ChchNPV	*C. chalcites*	L_5	0.1%	6.67-fold	Droplet	-	Bernal et al., 2014
			L_5	1%	40.21-fold	Droplet	-	Bernal et al., 2014
			L_6	0.1%	59.84-fold	Droplet	-	Bernal et al., 2014
			L_6	1%	142.56-fold	Droplet	-	Bernal et al., 2014
	HearNPV	*H. armigera*	L_3	0.1%	6.3-fold	Droplet	-	Ibargutxi et al., 2008
			L_3	1%	11.4-fold	Droplet	-	Ibargutxi et al., 2008
	SfMNPV	*S. frugiperda*	L_3	0.1%	1.32-fold	Droplet	-	Martínez et al., 2003
Leukophor AP ($C_{40}H_{42}N_{12}O_{10}S_2.2Na$)	ChchNPV	*C. chalcites*	L_2	1%	None	Droplet	-	Bernal et al., 2014
		C. chalcites	L_4	1%	2.02-fold	Droplet	-	Bernal et al., 2014
	SfMNPV	*S. frugiperda*	L_3	0.1%	1.42-fold	Droplet	-	Martínez et al., 2003
		S. frugiperda	L_4	0.1%	16-fold	Surface	-	Lasa et al., 2009
		S. frugiperda	L_4	1%	2640-fold	Surface	-	Lasa et al., 2009
	SeMNPV	*S. exigua*	L_4	0.1%	25.02-fold	Surface		Lasa et al., 2007
				1%	None	Field	Persistence OBs in surface	Lasa et al., 2007
			L_4	1%	5731-fold	Surface		Lasa et al., 2007
				1%	None	Field	Persistence OBs in surface	Lasa et al., 2007
Tinopal DMS	AgMNPV	*A.gemmatalis*	L_4	0.5%	35.7-fold	Diet	-	Morales et al., 2001
Tinopal LPW	AcMNPV	*A. californica*	L_2	1%	50-fold	-	-	Shapiro, 2000a
	AfMNPV	*Autographa falcifera*	L_2	1%	13-fold	Surface	-	Shapiro and Vaughn, 1995

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Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Tinopal LPW	AgMNPV	*A.gemmatalis*	L_4	0.05%	62.5-fold	Diet	-	Morales et al., 2001
	CfMNPV	*C. occidentalis*	L_4	1%	2-fold	Surface	LT_{50} decreased	Li and Otvos, 1999
	GmMNPV	*G. mellonella*	L_2	1%	2-fold	Surface	-	Shapiro and Vaughn, 1995
	HaMNPV	*H.armigera*	L_2	1%	25-fold	Surface	-	Shapiro and Vaughn, 1995
	HzSNPV	*H. zea*	L_2	1%	10-fold	Surface	-	Shapiro and Vaughn, 1995
	LdMNPV	*L. dispar*	L_2	1%	953.3-fold	-	-	Shapiro and Shepard, 2007
			L_2	1%	1670-fold	Surface	-	Shapiro and Robertson, 1992
			L_4	0.001%	1.09-fold	Surface	-	Shapiro and Robertson, 1992
			L_4	0.01%	2.50-fold	Surface	-	Shapiro and Robertson, 1992
			L_4	0.1%	118.40-fold	Surface	-	Shapiro and Robertson, 1992
			L_4	1%	184.70-fold	Surface	-	Shapiro and Robertson, 1992
	SeMNPV	*S.exigua*	L_2	1%	87.3-fold	Surface	-	Shapiro and Argauer, 2001
	SfMNPV	*S. frugiperda*	L_2	1%	115-fold	Surface	-	Martínez et al., 2000
	SfMNPV A	*S. frugiperda*	3-d-old	0.1%	87-fold	Surface	LT_{50} decreased	Hamm and Shapiro, 1992
	SfMNPV	*S. frugiperda*	L_3	1%	7.2-fold	-	-	Murillo et al., 2003
	SfMNPV	*S. frugiperda*	-	1%	None	Field	-	Hamm et al., 1994
			-	0.25%	Enhance mortality	Field	-	Hamm et al., 1994
	SfMNPV	*S. frugiperda*	3-d-old	0.1%	191-fold	Surface	LT_{50} decreased	Hamm and Shapiro, 1992
	SpliNPV	*S. littoralis*	L_3	1%	8.7-fold	-	-	Murillo et al., 2003

Table 1. Synergistic effects of optical brighteners on the insecticidal activity of baculoviruses.

Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Stilbene acid derivates								
Tinopal UNPA-GX ($C_{40}H_{44}N_{12}O_{10}S_2$)	ChchNPV	*C. chalcites*	L_4	1%	65.43-fold	Droplet	-	Bernal et al., 2014
			L_2	1%	4.43-fold	Droplet	-	Bernal et al., 2014
			L_3	1%	23.19-fold	Droplet	-	Bernal et al., 2014
	AgMNPV	*A gemmatalis*	L_4	0.5%	91-fold	Diet	-	Morales et al., 2001
	ChchNPV	*C. chalcites*	L_2	0.1%	0.54-fold	Droplet	-	Bernal et al., 2014
			L_3	0.1%	5.85-fold	Droplet	-	Bernal et al., 2014
			L_4	0.1%	7.35-fold	Droplet	-	Bernal et al., 2014
			L_5	0.1%	13.98-fold	Droplet	-	Bernal et al., 2014
			L_5	1%	70.41-fold	Droplet	-	Bernal et al., 2014
			L_6	0.1%	89.16-fold	Droplet	-	Bernal et al., 2014
			L_6	1%	397.13-fold	Droplet	-	Bernal et al., 2014
	HearNPV	*H. armigera*	L_3	0.1%	11.4-fold	Droplet	-	Ibargutxi et al., 2008
			L_3	1%	31.4-fold	Droplet	-	Ibargutxi et al., 2008
	MbrNPV	*M. brassicae*	L_2	1%		Diet	-	Mukawa and Goto, 2007
	SpltNPV	*S. litura*	L_4	1%	2188-fold	Droplet	PM disruption	Okuno et al., 2003
Tinopal UNPA-GX free acid ($C_{40}H_{42}N_{12}O_{10}S_2Na_2$)	ChchNPV	*C. chalcites*	L_2	1%	1.63-fold	Droplet	-	Bernal et al., 2014
			L_4	0.1%	2.46-fold	Droplet	-	Bernal et al., 2014

Table 1. Synergistic effects of optical brighteners on the insecticidal activity of baculoviruses.

Synergistic Agent	Baculovirus	Host insect	Insect instar	Synergistic Agent Concentration	Synergistic factor	Bioassay	Other effects	Reference
Styril-benzenic derivate								
Blankophor ER ($C_{24}H_{16}N_2$)	ChchNPV	*C. chalcites*	L_2	1%	None (1x)	Droplet	-	Bernal et al., 2014
		C. chalcites	L_4	1%	1.3-fold	Droplet	-	Bernal et al., 2014
	SfMNPV	*S. frugiperda*	L_3	0.1%	None	Droplet	-	Martínez et al., 2003
Pyrazoline derivate								
Hostalux SN ($C_{21}H_{26}ClN_3O_3S$)	ChchNPV	*C. chalcites*	L_2	1%	1.13-fold	Droplet	-	Bernal et al., 2014
			L_4	1%	2.13-fold	Droplet	-	Bernal et al., 2014
Biphenyl derivates								
Tinopal CBS	SfMNPV	*S. frugiperda*	L_3	0.1%	None	Droplet	-	Martínez et al., 2003
Benzimidazole derivate								
Uvitex BAC	SfMNPV	*S. frugiperda*	L_3	0.1%	0.3-fold	Droplet	-	Martínez et al., 2003
Benzoilureas derivate								
Clorfluazuron ($C_{20}H_9Cl_3F_5N_3O_3$)	AcMNPV	*S. exigua*	L_4	0.05 µg/larva	82.6-fold	Diet	LT_{50} decreased PM disruption	Guo et al., 2007a
Benzoato derivated								
Hostalux KSN ($C_{28}H_{16}N_2O_2$)	AgMNPV	*A gemmatalis*	L_4	0.5%	3.5-fold	Diet	-	Morales et al., 2001
Others								
Phorwite RKH ($C_{38}H_{38}N_{12}Na_2O_8S_2$)	LdMNPV	*L. dispar*	L_2	0.5%	1837-fold	Surface	-	Shapiro and Bell, 1982

Bioassays methods were done under laboratory conditions by surface contamination (Surface), droplet feeding method (Dropet) or inclusion in diet (Diet), and under field conditions (Field).

range is a difficult task. The host range of any virus is determined by its ability to enter the cells of susceptible hosts, and then to replicate and produce new infectious virus particles. In this process, the PM is not the only barrier to a productive infection and several mechanisms operate in conferring host cell specificity at different steps (Rohrmann, 2019).

A positive relationship between brightener concentration and the potentiation of nucleopolyhedrovirus pathogenicity has been established (Table 1) (Martínez et al., 2003; Shapiro and Argauer, 1997; Thorpe et al., 1999; Zou and Young, 1996). Tinopal LPW at 0.001% had no effect upon LdMNPV infectivity in its homologous host, however when the chemical concentration was increased, the potentiation also increased, from 2.5-fold at 0.001% to 185-fold at 1% (Shapiro and Robertson, 1992). Similarly, Tinopal UNPA-GX and Leucophor UO at 1% potenciated ChchNPV infectivity by 89.16 and 59.84-fold, while at 1% the potentiation was of 397.13 and 142.56-fold, respectively (Bernal et al., 2014). Leucophor AP at 1% was 229-fold more effective than at 0.1% (Lasa et al., 2007). There is also an instar-depending effect. The disruption effect is greater in advanced instars, usually more resistant to baculovirus infection than their younger counterparts (Table 1) (Hamm, 1999). Calcofluor white M2R at 0.1% enhanced SfMNPV LC50 to second instars by only 2.7-fold and by 61.4-fold in fourth instars (Martínez et al., 2003). Likewise, 1% of Leukophor UO and Tinopal UNPA-GX increased the ChchNPV activity in second instars by only 1.46 and 4.43-fold, respectively, and by 142.56 and 397.13-fold in sixth instars (Bernal et al., 2014). In young larvae, the PM is more porous and permable while, in advanced stages, it is well-formed, showing lower porosity (Erlandson et al., 2019; Peng et al., 1999; Wang and Granados, 2001).

Finally, the inoculation method clearly influences the enhancement of activity (Table 1). The highest increase has been observed when the inoculum is added to the diet by surface contamination (Lasa et al., 2009; Shapiro and Robertson, 1992). In contrast, when larvae were inoculated by the droplet feeding method, the observed enhancement was generally lower (Bernal et al., 2014) (Table 1). With the latter method, larvae ingest both the virus inoculum and the optical brightener in a short period of time (around 10 min), during which the chemical destroys the PM, facilitating the ODV entry to midgut cells. However, the destructive effect of optical brighteners

is transient and reversible and the PM recovers its integrity soon after consumption, as (Wang and Granados, 2001; Zhu et al., 2007). In contrast, upon surface contamination, larvae are constinuosly consuming virus inoculum, the effect of the chemical is prolonged over time, and a constant flow of ODVs throughout the PM is facilitated.

Because of the unique level of enhancement of viral infectivity by these optical brighteners on different lepidopteran pests under laboratory conditions, a patent for their use in biological control was awarded in 1992 (Shapiro et al., 1992). However, few field-tests have been performed and the enhacement activity is not yet evident under field conditions (Hamm et al., 1994; Lasa et al., 2007; Webb et al., 1994, 1996). Blancophor BBH increased LdMNPV infectivity by only 1.5-fold in forest plots (Webb et al., 1994) and inclusion of Tinopal LPW with SfMNPV increased *S. frugiperda* mortality by 12% (from 60% to 72%) (Hamm et al., 1994). Moreover, the enhancing effect observed under field conditions never reached that reported in the laboratory. The viral enhancer Blankophor BBH was applied by air to gypsy moth, *Lymantria dispar*, populations in two LdMNPV formulations ($2.5x10^{11}$ OBs/ha and $5x10^{11}$ OBs/ha) to determine if it could compensate for reduced virus rates. However, the addition of Blankophor BBH did not increase the efficacy of either formulation. In contrast, under laboratory conditions it reduced LC_{50} values by 213- and 314-fold on lettuce and oak, respectively (Thorpe et al., 1999). Similarly, laboratory results showed that addition of Leucophor AP reduced the lethal dosis of SeMNPV in fourth instars by 5,731-fold. Conversely, a reduction of crop defoliation in the greenhouses in Almería was not attained (Lasa et al., 2007). Calcofluor M2R increased 1,584 times the Pseudoplusia includens NPV infectivity under laboratory conditions, but when larvae were fed on soybean the pathogenicity was only 23% higher in fourth instars (Zou and Young, 1994). In addition to the poor field performance of brighteners, their high cost (Martínez et al., 2000) and their negative influence on crop growth, polinization and environment (Goulson et al., 2000, 2003) render them unacceptable for baculovirus formulations.

3. ENHANCINS

Enhancins are synergistic or viral enhancing factor, found firstly in GVs and later in a few NPVs and bacteria, with the ability to improve the infection of other baculoviruses (Biedma et al., 2015; Gallo et al., 1991; Goto, 1990; Guo et al., 2007b; Thiem, 2009; Tanada, 1956; Tanada et al., 1973). Enhancin genes have been identified in approximately 30% of NPVs and GVs sequenced to date. These genes are more common in GVs, present in about 46% of the genomes (Bivian-Fernández et al., 2012; Hayakawa et al., 1999; Li et al., 2021). In contrast, enhancin orthologues are found in a few Group II alphabaculoviruses, including Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) (Bischoff and Slavicek, 1997; Popham et al., 2001; Slavicek and Popham, 2005), Mamestra configurata nucleopolyhedrovirus (MacoNPV-A and MacoNPV-B) (Li et al., 2002a, 2002b), Choristoneura fumiferana MNPV (CfMNPV) (de Jong et al., 2005), and Agrotis segetum nucleopolyhedrovirus (AgseNPV-A) (Jakubowska et al., 2006). Several GVs and NPVs contain multiple enhancin genes, with XecnGV containing at least four. Enhancin genes have also been identified in the genomes of several bacteria including families Bacillales (Bacillaceae), *Enterobacterales (Yersinicaceae), Clostridiales (Clostriciaceae), Enterobacterales (Enterobacteriaceae)*, Bacillales (Listeriaceae) and the fungi Eurotiales (Trichocomaceae) (Ivanova et al., 2003; Parkhill et al., 2001; Read et al., 2003). However, their role as enhancers of infectivity has been poorly investigated.

3.1. Enhancins from baculoviruses

Betabaculovirus (GV) and alphabaculovirus (NPV) enhancins target IIM (Toprak et al., 2012; Wang and Granados, 1997). However, although both types of enhancins are similar, with sizesranging from 758 to 848 aa, and contain a conserved zinc-binding domain, characteristic of metalloproteases, they have different functions (Hoover et al., 2010; Slavicek, 2012). The presence of transmembrane domains in the carboxyl terminus of all NPV enhancins known to date suggests that their localization in ODV envelopes is a conserved feature of NPV enhancins (Slavicek, 2012). In contrast, GV enhancins are located within granules (Bischoff and Slavicek, 1997; Popham et al., 2001).

NPVs and GVs utilize distinct approaches to degrade the PM. GVs release a large amount of enhancin into the larval midgut, which then degrades the PM in a non-targeted random manner (Slavicek, 2012). In contrast, NPVs utilize enhancins located in ODV envelopes to “tunnel” through the PM to gain access to larval midgut cells and the enhancin activity is located on the site of interaction ODVs-PM. Therefore, degradation of the target IIMs by alphabaculovirus enhancins may be more subtle, than the prominent degradation caused by betabaculovirus enhancins (Slavicek, 2012). This has conditioned the synergism studies with both of them. Betabaculovirus enhancins have been extensively studied as enhancers of microbial infections, mostly by using purified enhancins from the granules or in mixed infections between GV and NPVs (Table 2). The highest rates of infection have been obtained with purified betabaculovirus enhancins (Table 2). In contrast, the presence of the NPV enhancins within the ODVs hinders their purification and their study as synergists of viral infection has been restricted to the construction of recombinant viruses.

3.1.1. Enhancins from betabaculoviruses

In an early study, a factor present in Pseudaletia unipuncta (Psun) GV (Lefavirales: Baculoviridae) was observed to increase the infectivity of PsunNPV OBs in mixed infections (Tanada, 1959). Later, Granados and collegues demonstrated that the enhancin from TnGV degraded major glycoproteins within the PM (Derksen and Granados, 1988; Lepore et al., 1996). To date, the enhancins of Agrotis segetum (Agse), Epinotia aporema (Epap), Helicoverpa armigera (Hear), Spodoptera frugiperda (Sf), Trichoplusia ni (Tn), Pseudaletia unipuncta (Psun) and Xestia c-nigrum (Xecn) GVs have been demonstrated to enhance baculovirus infections (Table 2) (Biedma et al., 2015; Gallo et al., 1991; Gizjen et al., 1995; Goto, 1990; Guo et al., 2007b; Jeyarani and Karuppuchamy, 2010; Lei et al., 2019; Tanada et al., 1975).

The enhancing effect of betabaculovirus enhancins has been demonstrated by different approaches (Table 2). Early studies showed that co-infection of GV OBs with NPV OBs significantly increased the infectivity and virulence of NPVs in their homologous hosts, in the absence of GV infection (Tanada, 1985; Tanada et al.,

1975; Tanada and Hukuhara, 1971). Thereafter, enhancins were realeased upon solubilisation of OBs in alkaline dissolution buffer, purified by size exclusion or gel filtration chromatography using mainly Sephacryl columns, and used together with NPVs. The viral-enhancing activity of purified betabaulovirus enhancins has been well-documented (Gallo et al., 1991; Gijzen et al., 1995; Guo et al., 2007; Lepore et al., 1996; Tanada, 1975; Wang et al., 1994). The high prevalence of this protein in the granules, of up to 5% (Erlandson et al., 2019; Gallo et al., 1991; Roelvink et al., 1995) facilitates its purification in high quantities. Alkaline soluble proteins of GV capsules have been postulated as promising additives for virus formulations and for *B. thuringiensis* toxin (Granados et al., 2001; Guo et al., 2019). Moreover, transgenic tobacco plants carrying betabaculovirus enhancins have been constructed and showed to sustain slower larval growth and development and increased larval susceptibility to baculovirus infection (Cao et al., 2002; Hayakawa et al., 2000, 2004; Mori et al., 2006).

Among the different betabaculovirus enhancins, TnGV enhancins have produced the highest improvement of baculovirus infections (Gallo et al., 1991; Wang et al., 1994). Reports of boosted infection by AcMNPV in mixtures with purified TnGV enhancins vary from a 60 - 63% increase in *T. ni* (Gallo et al., 1991; Lepore et al., 1996) to 82% in *Pseudaletia unipuncta* (Wang et al., 1994). Using recombinant viruses expressing the TnGV enhancin, the pathogenicity of NPV OBs increased by 39-fold for *S. exigua* larvae inoculated with AcMNPV, or by ten-fold for larvae inoculated with SeMNPV (Hayakawa et al., 2000). Larvae that ingested diet based on transgenic plants or mixtures of OBs and transgenic tobacco expressing enhancin showed increased susceptibility to infection (Cao et al., 2002; Mori et al., 2006). The delta-endotoxins of *Bacillus thuringiensis* were also more effective when formulated with TnGV enhancin (Granados et al., 2001; Guo et al., 2019).

Table 2. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhacin	Insect Pathogen	Host insect	Insect instar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Agrotis segetum GV (AsGV)								
Recombinant AcMNPV with enhancin fused to polyhedrin	AcMNPV	*S. exigua*	L_2	10^4 OBs/ml	Droplet	5.3-fold	-	Yang et al., 2017
	AcMNPV	*S. exigua*	L_2	Serial of concentrations	Droplet	5.7-fold	ST_{50} similar to WT OB yield similar to WT	Lei et al., 2019
		S. exigua	L_4	Serial of concentrations	Droplet	7.4-fold	OB yield similar to WT	Lei et al., 2019
Produced in procaryotic expression vector and purified by chromatography	HearSNPV	*H. armigera*	L_3	$1.17x10^2$ PIBs/ml	-	16.25%	-	Zhang et al., 2012
Epinotia aporema GV (EpapGV)								
Mixed infections	AgMNPV	*A.gemmatalis*	L_3	NPV: 50 OBs/larva GV: $6x10^4$ OBs/larva	Droplet	50%	ST_{50} decrease (7.7-5.7 days)	Biedma et al., 2015
			L_3	NPV: 300 OBs/larva GV: $6x10^4$ OBs/larva	Droplet	None	No differences in ST_{50}	Biedma et al., 2015
Helicoverpa armigera GV (HearGV)								
Mixed infections	LdMNPV	*H. zea*	L_2	-	Surface	300-fold	LT_{50} decreased by 18%	Shapiro, 2000b
	HearNPV	*H. armigera*	L_2 to L_5	NPV: LC_{25} for each instar GV: LC_{25} for each instar	Surface	Not ehnanced	TL_{50} increased	Jeyarani and Karuppuchamy, 2010
			L_2 to L_5	NPV: LC_{25} for each instar GV: LC_{50} for each instar	Surface	Not enhanced	TL_{50} increased	Jeyarani and Karuppuchamy, 2010
			L_2 to L_5	NPV: LC_{50} for each instar GV: LC_{25} for each instar	Surface	Not ehnanced	TL_{50} increased	Jeyarani and Karuppuchamy, 2010
			L_2 to L_5	NPV: LC_{50} for each instar GV: LC_{50} for each instar	Surface	Decrease infectvity of NPV	TL_{50} increased	Jeyarani and Karuppuchamy, 2010
Transgenic tobacco plants	-	*T. ni*	L_1	-	Feed on	-	30% lower weight 30% mortality	Cao et al., 2002

Table 2. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhancin	Insect Pathogen	Host insect	Insect instar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Pseudaletia unipuncta GV (PsunGV)								
Gel filtration chromatography	PsunNPV	*P. unipuncta*	L_5	Enhancin: $7.5x10^{10}$ capsules/ml NPV: $5x10^4$ OBs/ml	Diet	90%	-	Tanada et al., 1973
			L_5	Enhancin: $7.5x10^9$ capsules/ml NPV: $5x10^4$ OBs/ml (NPV)	Diet	65%	-	Tanada et al., 1973
			L_5	Enhancin: $7.5x10^8$ capsules/ml NPV: $5x10^4$ OBs/ml	Diet	30%	-	Tanada et al., 1973
			L_5	Enhancin: $5x10^9$ capsules/ml NPV: $5x10^6$ OBs/ml	Diet	90%	-	Tanada and Hukuhara, 1971
Mixed infections	P. separata EPV	*P. separata*	L_5	NPV: $2.1x10^8$ OB/larva EPV: $8.7X10^3$ Spheroids/larva	Diet	27-fold	-	Hukuhara et al., 2003
Spodoptera frugiperda GV (SfGV)								
Mixed infections	LdMNPV	*S. frugiperda*	L_2	-	Surface	13-fold	No differences in LT_{50}	Shapiro, 2000b
Trichoplusia ni GV (TniGV)								
Mixed infections	SeMNPV	*S. exigua*	L_3	GV: 10^7 OBs/ml NPV: $1.05x10^5$ OBs/ml	Diet	30-fold	-	Hayakawa et al., 2000
			L_3	GV: 10^7 OBs/ml NPV: $4.04x10^3$ OBs/ml	Diet	7-fold	-	Hayakawa et al., 2000
Recombinant AcMNPV	AcMNPV	*T. ni*	L_1	0.184 OBs/mm^2	Surface	2.18-fold	LT_{50} reduced by 17.4%	Del Rincón-Castro and Ibarra, 2005
Sf9 cells infected with recombinant AcMNPV	AcMNPV	*S. exigua*	L_3	Cells: 10^7 cells/ml NPV: $1.05x10^5$ OBs/ml (NPV)	Diet	39-fold	-	Hayakawa et al., 2000
	SeMNPV		L_3	Cells: 10^7 cells/ml NPV: $1.91x10^2$ OBs/ml (NPV)	Diet	10-fold	-	Hayakawa et al., 2000
Gel filtration chromatography	AcMNPV	*T. ni*	L_1	GV: 1 mg/ml NPV: $1x10^6$ OBs/ml	Surface	2-fold	-	Wang et al., 1994
			L_1	Enhancin: 40 µl column fraction NPV: $5x10^5$ OB/ml	Droplet	Increase	-	Gijzen et al., 1995
			L_1	GV: 50 pg/larva (GV) NPV: 0.3 OBs/larva	Droplet	Up to 60%	-	Lepore et al., 1996
			L_1	Enhancin: 10 mg/ml NPV: $1x10^5$ OBs/ml	Surface	63%	-	Gallo et al., 1991

Table 2. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhancin	Insect Pathogen	Host insect	Insect instar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Trichoplusia ni GV (TniGV)								
Gel filtration chromatography	AcMNPV	*T. ni*	L_1	Enhancin: 4.4 ng/larva NPV: $1.3x10^3$ puf/larva	Droplet	13%	-	Wang and Granados, 1998
			L_1	Enhancin: 1 ng/larva en NPV: $2.4x10^3$ puf/larva	Droplet	17%	-	Wang and Granados, 1998
			L_4	Enhancin: 1 ng/larva NPV: 5 OBs/larva	Surface	1.7-fold	-	Gallo et al., 1991
			L_4	Enhancin: 2.5 ng/larva NPV: 5 OBs/larva	Surface	2-fold	-	Gallo et al., 1991
			L_4	Enhancin: 5 ng/larva NPV: 5 OBs/larva	Surface	2.5-fold	-	Gallo et al., 1991
			L_4	Enhancin: 10 ng/larva NPV: 5 OBs/larva	Surface	3.4-fold	-	Gallo et al., 1991
			L_4	Enhancin: 20 ng/larva NPV: 5 OBs/larva	Surface	3.9-fold	-	Gallo et al., 1991
			L_4	Enhancin: 40 ng/larva NPV: 5 OBs/larva	Surface	4.5-fold	ST_{50} reduced	Gallo et al., 1991
			L_4	Enhancin: 1 µg/larva NPV: $1.5x10^6$ pfu/larva	Droplet	55%	-	Wang and Granados, 1998
			L_5	Enhancin: 1 µg/larva NPV: $1.5x10^6$ pfu/larva	Droplet	25%	-	Wang and Granados, 1998
			L_5	Enhancin: 0.2 µg/larva NPV: 10 OBs/ml	Diet	79%	-	Wang et al., 1994
		H. zea	L_1	Enhancin: 1 mg/ml NPV: $8x10^4$ OBs/ml	Diet	4-fold	-	Wang et al., 1994
			L_5	Enhancin: 1 µg/larva NPV: $2x10^6$ OBs/ml	Diet	10%	-	Wang et al., 1994
		P. unipuncta	L_1	Enhancin: 1 mg/ml NPV: $8x10^4$ OBs/ml	Diet	2-fold	-	Wang et al., 1994
			L_5	Enhancin: 1 µg/larva NPV: $2x10^6$ OBs/ml	Diet	82%	-	Wang et al., 1994
		S. exigua	L_1	Enhancin: 1 mg/ml NPV: $8x10^4$ OBs/ml	Diet	12-fold	-	Wang et al., 1994
			L_5	Enhancin: 1 µg/larva NPV: $4x10^6$ OBs/ml	Diet	13%	-	Wang et al., 1994

Table 22. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhancin	Insect Pathogen	Host insect	Insect instar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Trichoplusia ni GV (TniGV)								
Gel filtration chromatography	AgMNPV	*T. ni*	L_4	Enhancin: 2.5 ng/larva NPV: 40 OBs/larva	Surface	20%	ST_{50} reduced	Gallo et al., 1991
	TniSNPV	*T. ni*	L_4	Enhancin: 40 ng/larva NPV: 40 OBs/larva	Surface	38.9%	ST_{50} reduced	Gallo et al., 1991
			L_4	Enhancin: 1 µg/larva NPV: $1.3x10^5$ pfu/larva	Droplet	22%	-	Wang and Granados, 1998
			L_4	Enhancin: 1 µg/larva NPV: $3.3x10^5$ pfu/larva	Droplet	50%	-	Wang and Granados, 1998
	Bt toxins: Cry1Ac	*T. ni*	L_1	50 µg/ml	Droplet	≈30%	-	Fang et al., 2009
	Bt strain: Dipel	*T. ni*	L_1	Enhancin: 1 mg/ml *Bt*: 0.6 ng/larva	Droplet	6.4-fold	-	Granados et al., 2001
		H. zea	L_1	Enhancin: 1 mg/ml *Bt*: 5.1 ng/larva	Droplet	3.2-fold	-	Granados et al., 2001
		H. virescens	L_1	Enhancin: 1 mg/ml *Bt*: 0.7 ng/larva	Droplet	6-fold	-	Granados et al., 2001
	Dipel	*S. exigua*	L_1	Enhancin: 1 mg/ml *Bt*: 13.5 ng/larva	Droplet	4.4-fold	-	Granados et al., 2001
		P. includens	L_1	Enhancin: 1 mg/ml *Bt*: 0.4 ng/larva	Droplet	2.8-fold	-	Granados et al., 2001
		A. gemmatalis	L_1	Enhancin: 1 mg/ml *Bt*: 1.3 ng/larva	Droplet	5.8-fold	-	Granados et al., 2001
Transgenic rice plants	SeMNPV	*S. exigua*	L_2	Enhancin: 5% (w/w) leaf powder NPV: serial concentrations (LC_{50})	Diet	1.60-fold	-	Mori et al., 2006
	-	*S. litura*	L_2	Enhancin: 5% (w/w) leaf poder	Diet	-	-	Mori et al., 2006
	-	*P. separata*	L_2	Enhancin: 20% (w/w) leaf powder	Diet	-	Development inhibition	Mori et al., 2006
	AcMNPV	*S. exigua*	L_3	Enhancin: 0.5% (w/w) leaf powder NPV:serial concbnetration (LC_{50})	Diet	3-fold	-	Hayakawa et al., 2000
			L_3	Enhancin: 1.5% (w/w) leaf powder NPV: serial concentrations (LC_{50})	Diet	10-fold	-	Hayakawa et al., 2000

Table 3. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhacin	Insect Pathogen	Host insect	Insect instar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Trichoplusia ni GV (TniGV)								
Transgenic tobacco plants	SeMNPV	*S. exigua*	L_3	Enhancin: 0.5% (w/w) leaf powder. NPV: serial LD_{50}	Diet	2-fold	-	Hayakawa et al., 2000
				Enhancin: 1.5% (w/w) leaf powder. NPV: serial concetraction (LC_{50})	Diet	2-fold	-	Hayakawa et al., 2000
	-	*S. exigua*		Enhancin: 0.5% (w/w) leaf powder	Diet	-	Reduced body weight and developments	Hayakawa et al., 2004
		P. unipuncta						
	-	*T. ni*	L_1, L_3	Transgenic tocacco leafs	Leafs	-	Reduced body weight Larval mortality	Cao et al., 2002
Xestia c-nigrum GV (XecnGV)								
Gel filtration chromatography	XcnNPV	*X. c-nigrum*	L_4	Enhancin: 0-5 µg capsules NPV: serial concentrations	Diet	10-86%	-	Goto, 1990
	XcnNPV	*X. c-nigrum*	L_5	Enhancin: 0-40 µg capsules NPV: serial concentrations	Diet	10-80%	-	Goto, 1990
	SpliNPV	*S. litura*	L_3	Enhancin: 5 µg/ml capsules NPV: serial concentrations	Surface	1.25-fold	No differences in LT_{50}	Guo et al., 2007
		S. litura	L_5	Enhancin: 5 µg/ml capsules NPV: serial concentrations	Surface	6.48-fold	No differences in LT_{50}	Guo et al., 2007
	MabNPV	*M. brassicae*	L_2	0.1 mg/g diet NPV: serial concetration (LC_{50})	Surface	Up to 82%	-	Mukawa and Goto, 2007
Lymantria dispar MNPV (LdMNPV)								
En1 inactivation by mutagénesis	LdMNPV E1 inactived		L_4	Serial concetrations (LD_{50})	Surface	Up to 4-fold	-	Bischoff and Slavicek, 1997
Deletion by classical recombination	LdMNPV E1E2Del	*L.dispar*	L_2	Serial concetration (LD_{50})	Droplet	12-fold	-	Popham et al., 2001
	LdMNPV E1Del		L_2	Serial concetration (LD_{50})	Droplet	2.3-fold	-	Popham et al., 2001
	LdMNPVE2Del		L_2	Serial concetration (LD_{50})	Droplet	1.8-fold	-	Popham et al., 2001
	LdMNPV E1E2Del	*L.dispar* intact PM	L_4	-		14-fold	Function beyond PM degradation	Hoover et al., 2010
	LdMNPV E1E2Del	*L. dispar* disrupted PM	L_4			6-fold		Hoover et al., 2010

Table 2. Synergistic effects of enhancins produced by baculoviruses and entomopathogenic bacteria on the insecticidal activity of baculoviruses and *Bacillus thuringiensis* toxins

Source of enhacin	Insect Pathogen	Host insect	Insectinstar	Pathogen Concentration	Bioassay	Synergistic Factor	Other effects	Reference
Mamestra configurata NPV (MacoNPV)								
Bacmid tecnology	AcMNPV	*T. ni*	L_2	Serial concetration (LD_{50})	Diet	4.4-fold	ST_{50} reduced	Li et al., 2003
	-	*In vitro*	L_5	-	-	-	Disruption of PM IIM4 (target substrate specificity)	Toprak et al., 2012
Bacteria enhancin like (Bel) proteins								
Bacillus thuringiensis								
Expression in *E. coli*	Cry1Ac	*H. armigera*	L_5	3 µg/ml Cry1Ac 0.1 µg/ml Bel	Diet	52.5%	Degradation of PM mucin	Fang et al., 2009
				3 µg/ml Cry1Ac 0.8 µg/ml Bel	Diet	74.4%	Degradation of PM mucin	Fang et al., 2009
Bacillus anthracis								
Expression in *E. coli*	AcMNPV	*T. ni*	L_3	Did not enhance infection	Surface	Not differences	Cytotoxic effect	Galloway et al., 2005
Yersinia pestis								
Expression in *E. coli*	AcMNPV	*T. ni*	L_3	Did not enhance infection	Surface	Not differences	Cytotoxic effect	Galloway et al., 2005

ST_{50}, Median survival time. LD_{50}, Median lethal doses. DROPLET, Droplet-feeding method. SURFACE, Diet superficial incorporation. DIM, Diet incorporation method. WT, wild type. PIB, polyhedral inclusion body. Puf/larva, plaque-forming unit/larva.

3.1.2. Enhancins from alphabaculoviruses

The first alphabaculovirus *enhancin* gene that encodes a homolog of the GV enhancing was identified in LdMNPV, enhancin 1 (*E1*) or viral enhancing factor (*vef-1*), by chance during the mapping and sequencing of the gene mutated in LdMNPV few polyhedrin mutant (Bischoff and Slavicek, 1997). Inactivation of the enhancin gene through insertional mutagenesis reduced viral pathogenicity up to 4.0-fold (Bischoff and Slavicek, 1997). Later, complete genome sequencing revealed the presence of a second enhancin (*vef-2*). Single mutants decreased pathogenicitybetween 1.8 to 2.3-fold, while double mutants reduced it by 12-fold compared with wild type viruses (Popham et al., 2001). Both enhancins work collectively and are necessary for viral potency. A recombinant AcMNPV expressing the MacoNPV enhancin was 4.4-fold more pathogenic than parental viruses (Li et al., 2003). Later, it was shown that this recombinant was able to degrade IIM but exhibits target substrate specificity (Toprak et al., 2012). Moreover, NPV enhancin genes have a function beyond PM disruption, as LdMNPV double enhancin mutants were also less potent in larvae with a degraded PM (Hoover et al., 2010; Popham et al., 2001).

3.2. Enhancins from bacteria

Enhancin-like genes with a 24–25% nucleotide identity to viral *enhancins* have been found in *Yersinia pestis*, *Bacillus anthracis*, *B. cereus* and *B. thuringiensis* genome sequences (Galloway et al., 2005; Parkhill et al., 2001; Read et al., 2003). In addition, *enhancin* genes have also been identified in the genomes of *Salmonella enterica*, *Clostridium perfringens*, *Aspergillus oryzae*, *Enterobacter aerogenes* and *Listeria ivanovii*. It seems that bacteria may have acquired *enhancin* genes from baculoviruses by horizontal gene transfer. The *enhancin* genes from bacteria could be obtained by an exchange of genetic material between the ancestor of *B. cereus* residing in the insect gut and the NPVs ingested, (Slavicek and Popham, 2005). Furthermore, the *enhancin* gene of *Y. pestis* is flanked by a tRNA gene and transposase fragments, which may suggest that this bacterium obtained its *enhancin* gene by horizontal transfer. A typical metalloprotease zinc-binding domain (HEIAH) is also present in bacterial enhancins, mpbE (metalloprotease bacillus *enhancin*)

(Hajaij-Ellouze et al., 2006). However, their role in bacterial infection is not so clear. A first study concluded that bacterial enhancins might have distinct biochemical function as they were cytotoxic compared to viral enhancin and they did not increase viral infection in the same way as viral enhancins did (Galloway et al., 2005). In contrast, a later study assesses that Bel protein from *B. thuringiensis* augmentates the toxicity of Cry1Ac up to 74% by degrading the IIM of *H. armigera* and *T. ni* larvae (Fang et al., 2009). There are no more studies on the enhancing activity of Bel proteins.

4. CHITINASES

Chitinolytic enzymes have aroused considerable interest as defensive agents against chitin-containing pestiferous and pathogenic organisms, such as insects, nematodes, and fungi (Berini et al., 2018; Carr and Klessig, 1989; Linthorst, 1991; Sahai and Manocha, 1993). Crude chitinases have been extensively reviewed as biological control agents (Berini et al., 2018; Hartl et al., 2012; Herrera-Estrella and Chet, 1999; Kramer and Muthurkrishnan, 1997; Oyeleye and Normi, 2018; Rathore and Gupta, 2015; Singh et al., 2014; Singh and Arya, 2019). They have also been exploited as chemical defense proteins against fungal pathogens and herbivore insects in transgenic plants (Corrado et al., 2008; Di Maro et al., 2010; Fiandra et al., 2010; Herrera-Estrella and Chet, 1999). Chitinases target chitin and disturb PM structure and permeability, causing perforations that lead to disruption in larval growth and development, and even death (Berini et al., 2016; Brandt et al., 1978). The chitinase from AcMNPV (ChiA) produced in an *E. coli* expression system induces perforations on the PM that increase PM permeability (Rao et al., 2004). Therefore, they have been postulated as synergistic agents that facilitate the entry of pathogens into susceptible insects tissues.

Only one study has described chitinolytic enzymes as synergistic products for baculoviruses. As early as 1987, Shapiro and coworkers demostrated that the addition of 1% commercial bacterial chitinase to LdMNPV suspensions reduced the LC_{50} up to 5.4-fold, and larvae died more rapidly (Shapiro et al., 1987). However, to date there are no more studies on the use of purified chitinases as enhancers of baculovirus infections.

In contrast, the effect of recombinant chitinases expressed on viruses or plants has received more attention. A recombinant AcMNPV expressing the *Manduca sexta* (Lepidoptera: Sphingidae) chitinase was 75% more virulent that the parental virus (Gopalakrishnan et al., 1995). Similarly, a recombinant AcMNPV that expressed the ChiA from SpliNPV showed a reduced chitiobiosidase and endochitinase activity compared with wild-type virus, which led to increased insecticidal activity by reducing the LD_{50} up to 7-times and the LT_{50} by 88 h (Wang et al., 2013). Moreover, due to the increased PM permeability it produces, AcMNPV ChiA has been proposed as a gut permeation enhancer in oral delivery strategies of bioinsecticides targeting haemocoelic receptors (Fiandra et al., 2010). Transgenic tobacco plants expressing chitinases from different organisms such as AcMNPV, *M. sexta*, *Drosera rotundifolia* L. (Caryophyllales: Droseraceae) or *Trichoderma harzianum* Rifai (Hypocreales: Hypocreaceae), showed enhanced resistance to biotic and abiotic stress agents and reduced damage caused by fungi and lepidopteran larvae (Corrado et al., 2008; Dana et al., 2006; Di Maro et al., 2010; Ding et al., 1998; Durechova et al., 2019; Wang et al., 1996).

Finally, chitinolytic enzymes from bacteria and insects have been largely tested in combination with other microbial pathogens such as *Bt* spore-crystal supensions (Kramer and Muthukrishnan, 1997). In these assays, larvae were generally more susceptible to *Bt* toxins. Chitinolytic bacterial strains isolated from lepidopteran midguts were able to increase the activity of *Bt* spore-crystals (Sneh et al., 1983). Crude chitinase preparations from *B. circulans* enhanced the toxicity of *Bt* var. kurstaki against diamondback moth larvae (Wiwat et al., 1996). Several *B. thuringiensis* strains with chitinolytic activities were 2.35-fold more effective than control *Bt* strains (*Bt* strain DL5789) against *S. exigua* larvae (Liu et al., 2002). Larvae of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), fed on artificial diet containing 2% transgenic tobacco plant expressing the *M. sexta* chitinase were more susceptible to *Bt* toxins (Ding et al., 1998).

Table 3. Effects of chitinases produced by different organisms on the insecticidal activity of baculoviruses and/or on PM disruption.

Source of chitinase	Production method	Baculovirus	Host insect	Insect instar	Chitin concentration	Synergistic factor	Other effect	Reference
Commercial								
	Commercial	LdMNPV	*L. dispar*	L_2	0,001%	1.3-fold	-	Shapiro et al., 1987
					0,01%	2-fold	-	Shapiro et al., 1987
					0,10%	3.2-fold	-	Shapiro et al., 1987
					1%	5.4-fold	-	Shapiro et al., 1987
AcMNPV ChiA								
	Expressed in *E.coli* and purified by chromatography	-	*Bombyx mori* (Lepidoptera: Bombycidae)	L_5	0.75 µg/g larva	-	PM disruption	Rao et al., 2004
	Expressed in transgenic plants and purified by chromatography	-	*Alternaria alternata* (Fungus)	-	75 µg/ml	-	Inhibition of spore germination	Di Maro et al., 2010
		-	*B. mori*	L_5	50 µg/ml	-	PM disruption	Di Maro et al., 2010
		-	*H. virescens*	L_5	40 µg/ml	-	PM disruption	Di Maro et al., 2010
	Transgenic tobacco plants	-	*H. virescens*	L_4	-	-	PM disruptionweight reduction	Fiandra et al., 2010
			H. virescens		-	-	PM disruption Reduced plant damage	Corrado et al., 2008
Manduca sexta ChiA								
	Recombinant bacmid	AcMNPV	*S. frugiperda*	L_4	$2x10^5$ OB/ml	1,3-fold	-	Gopalakrishnan et al., 1995
Epinotia aporema GV (EpapGV) ChiA								
	Recombinant bacmid	AcMNPV	*A. gemmatalis*	-	-	-	PM disruption	Salvador and Ferrelli, 2014
SpliNPV ChiA								
	Recombinant bacmid	AcMNPV	*S. exigua*	L_3	10^4 OB/ml	7-fold	-	Wang et al., 2013

5. CONCLUSIONS

The high cost associated with the baculovirus mass production system is one of the greatest limitations for the use of baculovirus or other microbial pathogens in pest control (Grzywacz et al., 1998; Gupta et al., 2007; Hunter-Fujita, 1998). For this reason, coadjuvants that allow reductions in the amount of active compounds in bioinsecticide formulations have been the subject of intensive research. The present review aims to evaluate the enhancing effect of chemical or natural PM disrupting agents given their potential as synergistic agents in baculovirus formulations or for other *per os* infectious pathogens. Incorporation of such synergistic substances to baculovirus or microbial formulations allows reduction in the amount of active matter, making baculovirus formulations more competitive with chemical insecticides, Among the different disruption agents, optical brighteners produced by far the highest enhancement of baculovirus infection. These are chemical compounds with potent chiting binding properties that displace the structural peritrophins from the PM, affecting chitin biosynthesis (Wang and Granados, 2001). However, their chemical origin is not considered rational and environmentally friendly in biological control products. In contrast, naturally occurring enhancins and chitinases can be part of bioinsecticides. They are more specific and target the mucin and quitin PM components, respectively. Moreover, enhancins degrade major structural PM proteins but exibit target substrate specificity (Toprak et al., 2012). Therefore, if proper formulations can be made purified enhancins and chitinases have the potential to increase the effectiveness of entomopathogenic viruses for the biocontrol of certain lepidopteran pests of forestry and agriculture.

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CHAPTER III

Bacmid Expression of Granulovirus Enhancin En3 Accumulates in Cell Soluble Fraction to Potentiate Nucleopolyhedrovirus Infection

ABSTRACT

Enhancins are metalloproteinases that facilitate baculovirus infection in the insect midgut. They are more prevalent in granuloviruses (GVs), constituting up to 5% of the proteins of viral occlusion bodies (OBs). In nucleopolyhedroviruses (NPVs), in contrast, they are present in the envelope of the occlusion-derived virions (ODV). In the present study, we constructed a recombinant Autographa californica NPV (AcMNPV) that expressed the Trichoplusia ni GV (TnGV) enhancin 3 (En3), with the aim of increasing the presence of enhancin in the OBs or ODVs. En3 was successfully produced but did not localize to the OBs or the ODVs and accumulated in the soluble fraction of infected cells. As a result, increased OB pathogenicity was observed when OBs were administered in mixtures with the soluble fraction of infected cells. The mixture of OBs and the soluble fraction of Sf9 cells infected with BacPhEn3 recombinant virus was ~3- and ~4.7-fold more pathogenic than BacPh control OBs in the second and fourth instars of *Spodoptera exigua*, respectively. In contrast, when purified, recombinant BacPhEn3 OBs were as pathogenic as control BacPh OBs. The expression of En3 in the soluble fraction of insect cells may find applications in the development of virus-based insecticides with increased efficacy.

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1. INTRODUCTION

Enhancins are virus-encoded metalloproteinases that can enhance the establishment of baculovirus infection by degrading the peritrophic matrix (PM) in the midgut of the host insect. They act as a protease and degrade the insect intestinal mucin (IIM), the major mucinous protein that constitutes the PM, resulting in the disruption of this structure and increasing its permeability to baculovirus occlusion-derived virions (ODVs) (Derksen and Granados, 1988; Erlandson et al., 2019; Wang et al., 2014; Wang and Granados, 1997, 2001; Wang and Hu, 2019).

Enhancin genes are prevalent in lepidopteran-infecting granuloviruses (GV, genus *Betabaculovirus*), where they are present in the occlusion body (OB) matrix (Erlandson et al., 2019; Gallo et al., 1991). The virus-enhancing activity of betabaculovirus enhancins has been well-documented (Erlandson et al., 2019; Gallo et al., 1991; Gijzen et al., 1995; Guo et al., 2007; Lepore et al., 1996; Tanada et al,. 1973; Wang et al., 1994). In contrast, orthologues present in lepidopteran-infecting nucleopolyhedroviruses (NPV, genus *Alphabaculovirus*) are associated with ODV envelopes and their efficacy as enhancers of NPV infections has come from experiments with recombinant viruses (Bischoff and Slavicek., 1997; Erlandson et al., 2019; Li et al., 2003; Slavicek and Popham., 2005). Some betabaculovirus and alphabaculovirus genomes contain multiple copies of these genes (Bischoff and Slavicek., 1997; Bivian-Hernández et al., 2017; Li et al., 2003; Slavicek and Popham., 2005).

Most of the studies on infection potentiation by betabaculovirus enhancins have been performed using enhancins purified from OBs (Lepore et al., 1996; Gallo et al., 1991; Gijzen et al., 1995; Guo et al., 2007; Tanada et al,. 1975; Wang et al., 1994), whereas others have employed bacmid technology to express enhancin genes in recombinant NPVs (Del Rincón-Castro and Ibarra, 2005; Hayakawa et al., 2000; Lepore et al., 1996; Lei et al., 2019; Yang et al., 2017). One question concerning the recombinant enhancin expressed by an NPV is whether the recombinant enzyme is occluded within OBs, as in GVs, or integrated into the ODV envelope, as occurs in NPVs. Previous studies in which the Trichoplusia ni GV (TnGV) enhancin gene was expressed in recombinant Autographa californica NPV (AcMNPV) did not specifically

address the localization of the recombinant protein (Del Rincón-Castro and Ibarra, 2005; Hayakawa et al., 2000; Lepore et al., 1996). For example, TnGV enhancin purified from cells infected by a recombinant AcMNPV effectively degraded PM proteins and increased lethal infection in host larvae (Lepore et al., 1996). Others have suggested that the enhancin may be occluded within OBs, as recombinant OBs were significantly more pathogenic than wild-type OBs in larval bioassays (Del Rincón-Castro and Ibarra, 2005; Hayakawa et al., 2000).

Interestingly, the incorporation of a betabaculovirus enhancin into alphabaculovirus OBs was achieved by fusing the enhancin gene to a C-terminal section of the polyhedrin gene (Lei et al., 2019; Yang et al., 2017). Using this technology, a recombinant AcMNPV bacmid was constructed that embedded both Agrotis segetum GV enhancin and the Cydia pomonella GV GP37 enhancing factor within the OBs (Lei et al., 2019). The GP37 enhancing factor is homologous to fusolin found in entomopoxviruses that binds chitin and enhances viral infection (Takemoto et al., 2008). These recombinant viruses produced normal OBs that were significantly more pathogenic to susceptible larvae than wild-type OBs (Lei et al., 2019; Yang et al., 2017). It is unclear, however, whether enhancin can be occluded into OBs or enveloped in the ODVs of an NPV without the use of the polyhedrin fusion technique.

The main objective of the present study was to determine whether an unmodified betabaculovirus enhancin, not fused with polyhedrin, was incorporated within NPV OBs or enveloped in the ODVs. For this, a polyhedrin-positive recombinant AcMNPV expressing a betabaculovirus enhancin was constructed. The localization of the recombinant protein was investigated by SDS-PAGE analyses and the insecticidal activity of the recombinant OBs was compared with that of wild-type OBs.

2. MATERIAL AND METHODS

2.1. Insect, cells and viruses

Larvae of *Spodoptera exigua* were obtained from a healthy laboratory colony maintained on a semi-synthetic diet (Greene et al., 1976) at 25 ± 1 °C. Sf9 cells (ThermoFisher Scientific, Waltham, MA, USA) were maintained in TC100 medium (Lonza Bioscience, Cologne, Germany) supplemented with 10% fetal calf serum

(Lonza Bioscience, Cologne, Germany) at 28 ± 1 °C (King and Possee, 1992). For the construction of the recombinant virus expressing the enhancin gene, the Bac-to-Bac™ Baculovirus Expression System was used (ThermoFisher Scientific, Waltham, MA, USA). The enhancin gene was amplified from TnGV (Bivian-Hernández et al., 2017), whereas the polyhedrin gene was amplified from the AcMNPV C6 clone, the type species of the *Alphabaculovirus* genus (Ayres et al., 1994). All viruses were obtained from the virus collection of the Microbial Bioinsecticides group at the Universidad Pública de Navarra.

2.2. Recombinant BacPh and BacPhEn3 virus DNAs

We selected enhancin 3 (En3) of TnGV to construct the recombinant virus as TnGV-purified enhancins produced the highest potentiation (discussed in Appendix A). Among the three enhancins present in the TnGV genome (Bivian-Hernández et al., 2017), En3 possesses both the zinc-binding domain of metalloproteinases and the mucin-binding domain (Figure S1) (explained in Appendix B).

The recombinant virus was constructed using a Bac-to-Bac recombination system (ThermoFisher Scientific, Waltham, MA, USA) (Luckow et al., 1993). Two primer sets were designed to amplify the full-length coding sequence (from ATG to stop) of the enhancin 3 gene (*en3*, viral enhancing factor 3) of TnGV (TnOrf-149; nt 153,610 to 156,315 in the TnGV genome; accession number KU752557 or NC_038375), and the polyhedrin gene (*ph*) of AcMNPV (AcOrf-8; nt 4520 to 5257 in the AcMNPV-C6 genome; accession number L22858 or NC_001623) (Table 1). We did not include a His-tag sequence in the C-terminal for protein purification and detection, in order to produce a natural enhancin and to determine its natural location. Previously, the use of an N-terminal His-tag for identification and purification purposes resulted in reduced stability and binding properties of tagged proteins (Booth et al., 2018), and compromised the mucin degradation activity of the tagged enzyme in the case of enhancin-like Bel proteins from bacteria (Galloway et al., 205). The genes were cloned in the pFastBacTM Dual (pFBD-phx-p10x) expression vector under the polyhedrin (*en3* gene) and p10 (*ph* gene) promoters. For the insertion of the specific genes, two pairs of primers were designed to amplify the *en3* gene of TnGV and *ph* of AcMNPV. The *Xba*I and *Pst*I restriction sites were introduced near

the 5′ termini of the forward and reverse primers of *en3*, respectively, for further cloning and to direct the transcription of the *en3* with the polyhedrin promoter. Additionally, downstream from the *Xba*I restriction site and upstream from the ATG codon, a 20 nt sequence was inserted that corresponded to the *en3* promoter region, in order to transcribe this gene under its homologous promoter (Lepore et al., 1996). Similarly, for the *ph* gene, the *Xho*I and *Nco*I restriction sites were introduced near the 5′ termini of the forward and reverse primers, respectively, to direct cloning of the p10 promoter. The forward primer (Ph-Fw) was designed 308 nt upstream of the ATG codon, to avoid an AT rich region (69% AT and 31% GC), as baculovirus intergenic regions are AT rich and the primer could otherwise anneal at different points across the genome.

Table 1. Primers used in this study.

Primer	Sequence	Amplification Purpose
En3-TnGV-Fw	5′-TC**TCTAGA**GCTGCATTAATTATAAGACTATGTC -3	En3 amplification. TnGV DNA was used a template. In bold—the *Xba*I site. Underlined En3 promoter and ATG start codon; nt 153,590 to 153,614 in the TnGV genome. Tm 51 °C.
En3-TnGV-Rv	5′-CC**CTGCAG**TTAGAACGCTATCATTTTTAACG-3	En3 amplification. TnGV DNA was used a template. In bold—the *Pst*I site. Underlined—the *en3* TAA stop codon; nt 156,293 to 156,315 in TnGV genome. Tm 50 °C.
Ph-Fw	5′-CG**CTCGAG**GCCGGCATAGTACGC-3′	Ph amplification. AcMNPV C6 DNA was used a template. In bold—the *Xho*I site; nt 4197 to 4211 in AcMNPV genome. Tm 56 °C.
Ph-Rv	5′-CG**CCATGG**TTAATACGCCGGACCAGTG-3′	Ph amplification. AcMNPV C6 DNA was used a template. In bold—the *Nco*I site. Underlined—the *ph* TAA stop codon; nt 5239 to 5257 in AcMNPV genome. Tm 54 °C.
En3-Seq-Fw	5′-CCGTACCCGCAAATATG-3′	En3 sequence confirmation. Primer that annealed at nt 1053 to 1073 in the *en3* gene. Tm 53 °C.
pFBD-seq-Fw	5′-CCGTGTTTCAGTTAGCC-3′	En3 sequence confirmation. Primer that annealed at nt 7563 to 7579 in pFBD plasmid. Tm 54 °C.
M13-Fw	5’-CCCAGTCACGACGTTGTAAAACG-3´	For confirmation of correct insertion of *en3* and *ph*. Primer that flanked the mini att-Tn7 site of the bacmid. Tm 54 °C.
M13-Rv	5´-AGCGGATAACAATTTCACACAGG-3´	For confirmation of correct insertion of *en3* and *ph*. Primer that flanked the mini att-Tn7 site of the bacmid. Tm 53 °C.

PCR amplifications were conducted using the Phusion high-fidelity Pfu DNA polymerase (New England Biolabs, Ipswich, MA, USA) and amplicons were recovered by using the PCR clean-up extraction kit NucleoSpin® Extract II Kit (Macherey-Nagel, Düren, Germany). The *en3*- and *ph*-purified products were then ligated into pJET1.2/blunt plasmid (CloneJET PCR Cloning Kit, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Ligation mixtures were transformed into *Escherichia coli* XL1-Blue electrocompetent cells using standard procedures. Positive clones were identified by colony-PCR. Five positive clones were grown, and plasmid DNAs were purified using the NucleoSpin R Plasmid Kit (Macherey-Nagel Inc., Düren, Germany). Subsequently, two selected plasmids for each gene were sequenced by ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kits and an ABI PRISM 3100 Genetic Analyzer (STABVida, Caparica, Portugal). After sequence confirmation, a selected clone was digested with *Xba*I and *Pst*I enzymes and electrophoresed in 1% agarose gel. The generated restriction fragment was extracted from the gel and purified by column and ligated into *Xba*I-*Pst*I-digested pFBD vector to generate the pFBD-En3. Two independent clones were selected for sequencing as described above. Once the correct insertion of *en3* was confirmed, the *ph* gene was cloned in the pFBD-En3 by double digestion with *Xho*I-*Nco*I to obtain the recombinant plasmid pFBD-PhEn3, under the control of the p10 promoter. Two independent clones were selected for sequencing to confirm the correct insertion of the *ph* gene. Finally, ligation products were electroporated into *E. coli* DH10BacTM cells (ThermoFisher Scientific, Waltham, MA, USA) that contained the AcMNPV shuttle vector (bacmid) and the helper plasmid, to produce the recombinant bacmid BacPhEn3, following transposition of the pFBD-PhEn3 expression construct through the transposition of site-specific cassettes between Tn7R and Tn7L and the subsequent steps following standard protocols. In parallel, a negative control virus was constructed that included the *ph* gene without any gene of interest, to generate the pFBD-Ph vector, previously digested with *Xho*I-*Nco*I enzymes, under the *p10* promoter, following the same procedure.

Colonies were selected on LB (Luria Bertani) agar plates containing kanamycin (50 µg/mL), gentamycin (7 µg/mL), tetracycline (10 µg/mL), X-gal (100 µg/mL) and

IPTG (40 µg/mL). Ten white colonies were re-streaked on fresh LB agar plates under the same conditions to avoid contamination. Recombinant bacmid DNA was isolated using the PureLinkTM HiPure Plasmid DNA Miniprep Kit (ThermoFisher Scientific, Waltham, MA, USA). The successful transposition to bacmid was confirmed by digestion of DNA with *Pst*I and by PCR amplification and sequence analysis using M13-Fw and M13-Rv primers that annealed outside the coding region of the *p10* and *ph* promoters in the pFBD vector (Table 1).

2.3. Transfection of Sf9 cells with recombinant BacPh and BacPhEn3 virus DNAs

Recombinant baculoviruses were produced in a 25 cm^2 cell culture flask by transfecting 1 µg of BacPhEn3 and BacPh genomic DNAs into 10^6 Sf9 cells using Lipofectin® Reagent (ThermoFisher Scientific, Waltham, MA, USA) (King and Possee, 1992; O'Reilly et al., 1994). The resulting OBs of both recombinant viruses were compared with those produced by the wild-type AcMNPV C6. For this, 10^6 Sf9 cells were inoculated with AcMNPV-C6 budded virions (BVs) at a m.o.i. of 10 that we had in stock. The production of OBs in cells was checked daily, and five days post-infection, the supernatant containing BVs and the pellet containing the cells and viral OBs were recovered by centrifugation (2400× *g*, 5 min). DNA extraction was performed on infected cells (King and Possee, 1992) to confirm the restriction endonuclease (REN) profiles of the recombinant bacmids and to check for cross-contamination. Finally, to evaluate the normal occlusion of ODVs within the OBs of the two recombinants, the number of infectious units within OBs was compared with that of wild-type virus AcMNPV-C6 by end-point dilution in Sf9 cells (King and Possee, 1992; O'Reilly et al., 1994). Specifically, at five days post-infection, OBs were released from cells by sonication and OBs were counted in triplicate samples in a Neubauer hemocytometer. A 500 µL volume of suspension containing 10^8 OBs/mL was mixed with an equal volume of 0.1 M Na_2CO_3 and stirred at 28 °C for 30 min. Undissolved OBs and cell debris were removed by centrifugation (5900× *g*, 5 min), and the supernatant containing the ODVs was filtered through a 0.45 µm filter and used in the end-point dilution assay (Greene et al., 1976; Luckow et al., 1993).

2.4. Production of BacPh and BacEn3 OBs in larvae

The BacPh and BacPhEn OBs were produced in larvae by injecting the BV suspension obtained from transfection assays. For this, BV suspensions were initially quantified by plaque assay (King and Possee, 1992; O'Reilly et al., 1994) and both suspensions had similar titers (3.51×10^7 pfu/mL for BacPh and 3.80×10^7 pfu/mL for BacPhEn3; $t = 2.139$; d.f. = 6; $p = 0.076$). Therefore, recombinant bacmid OBs were amplified by injecting the same quantity of BVs of BacPh and BacPhEn3, 8 µL of BV suspension (1:1000), into *S. exigua* fifth instar larvae from the laboratory colony. Groups of 25 larvae were inoculated with each virus and were reared individually in 30 mL plastic cups with a piece of semi-synthetic diet. Virus-killed larvae were collected daily and transferred to a 50 mL collection tube. OBs were purified from dead larvae by filtration through muslin and several rounds of centrifugation in 0.1% (wt/vol) sodium dodecyl sulfate (SDS) at 2400× *g* 5 min. Finally, OBs were resuspended in 1 mL double-distilled water and stored at 4 °C until required. The fidelity of OBs produced in insects was confirmed by REN analysis and by sequencing of the PCR products obtained following amplification using M13-Fw and M13-Rv primers (Table 1).

2.5. Detection and localization of En3 protein

To detect and localize the TnGV En3 protein an SDS-PAGE was performed using different samples of cells and OBs produced both in cell culture and in larvae. The protocol to obtain the different samples was as follows. A 25 cm^2 cell culture flask containing a batch of 10^6 Sf9 cells was transfected with the DNAs of BacPh and BacPhEn3, as mentioned in Section 2.3. At five days post-infection, the Sf9 cells and medium were recovered with a cell scraper (Bio-Rad, Berkeley, CA, USA). To separate the medium containing the BVs from the cells containing the OBs, a low-speed centrifugation was performed at 2400× *g* during 5 min. The supernatant sample containing the BVs was transferred to a 15 mL collection tube (Sample 1; S1). The pellet with the cells and OBs were rinsed twice with 1 mL phosphatase-buffered saline (PBS) pH 7.4 and resuspended in 1 mL PBS. OBs were released from cells by sonication in an ultrasonic bath (Selecta Master, JP Selecta, Abrera, Spain) for 10 min at maximum power (100 W) to produce sample 2 (S2). The lysate was then centrifuged at 5900× *g* for 5 min. The pellet containing the cell debris and OBs was rinsed twice with 500 µL of PBS and resuspended in 500 µL PBS to produce

sample 3 (S3). The supernatant containing the soluble phase of the infected cells was transferred to a 1.5 mL microcentrifuge tube to produce sample 4 (S4). The OBs produced in larvae and semi-purified by filtration through muslin and differential centrifugation (2400× *g* during 5 min) were also analyzed as sample 5 (S5). Three replicates were performed in parallel.

For each sample (S1–S5) and virus, an aliquot of 20 µL was mixed with the same volume of 2× sample buffer (Bio-Rad, Berkeley, CA, USA), boiled at 100 °C for 5 min, and then subjected to electrophoresis as previously described (Laemmli, 1970), using Criterion TGX™ 4–20% Precast Gel (Bio-Rad, Berkeley, CA, USA). Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Berkely, CA, USA) and then distained in 30% ethanol and 10% acetic acid and photographed.

2.6. Insect bioassays

To determine the insecticidal characteristics and the enhancement activity of BacPolhEn3 recombinant OBs, insect bioassays were performed in second and fourth instar larvae of *S. exigua*. OBs originated from lysate cell culture and the OBs were produced in larvae. OBs from lysate cultures were used directly without any purification (OBs and cell medium), whereas those produced in larvae were purified by filtration and several rounds of centrifugation. Triplicate samples of OBs were counted in a Neubauer hemocytometer.

A discriminating concentration assay was initially performed using two concentrations of OBs for each instar. Second instars were inoculated with 2×10^4 and 2×10^5 OBs/mL, whereas fourth instars were inoculated with 10^5 and 10^6 OBs/mL using the droplet feeding method (Hughes and Wood, 1981). Larvae were starved for 8 to 12 h at 25 ± 1 °C and then allowed to drink from an aqueous suspension of OBs and 10% sucrose plus 0.001% (*w*/*v*) Fluorella Blue. Control larvae were treated identically but did not consume OBs. Groups of 28 larvae that ingested the suspension within 10 min were transferred individually to the wells of a 28-well plate with a piece of semi-synthetic formaldehyde-free diet (Greene et al., 1976). Larvae were reared at 26 ± 1 °C and mortality was recorded daily until insects had either died or pupated. Bioassays were performed five times (replicates) using different

batches of larvae. Virus-induced mortality results were analyzed within each instar and each OB concentration, to compare the OBs from cell culture lysate with those purified from larvae. A Shapiro–Wilk test and Levene's test indicated that the data were normally distributed with homogeneity of variance. The results were then subjected to three-way analysis of variance (ANOVA) with virus (BacPh and BacPhEn3), origin (OBs + cell lysate and OBs from larva), and inoculum concentration (high and low) specified as factors. Means were compared by Tukey test. The analysis was performed in the R-based package Jamovi v.1.2.27.0 (Jamovi, 2020).

The pathogenicity of BacPhEn3 OBs produced in cell culture and in larvae was compared with that of BacPh OBs in concentration-mortality bioassays in second and fourth instars of *S. exigua*. Bioassays were performed following the droplet feeding method (Hughes and Wood, 1981) to inoculate larvae with one of five concentrations of OBs. For second instars, the concentrations were 2×10^7, 4×10^6, 8×10^5, 1.6×10^5 and 3.2×10^4 OBs/mL, whereas for fourth instars, 1×10^8, 1×10^7, 1×10^6, 1×10^5 and 1×10^4 OBs/mL were used to inoculate larvae. These concentrations were previously determined to kill between 95% and 5% of the experimental insects. Control larvae were treated identically but did not consume OBs. Groups of 28 larvae that ingested the suspension within 10 min were reared individually at 26 ± 1 °C and mortality was recorded daily until insects had either died or pupated. The bioassay was performed three times using different batches of larvae. Virus-induced mortality results were subjected to probit analysis using the Polo-Plus program (LeOra, 1987).

3. RESULTS

3.1. Recombinant BacPh and BacPhEn3 virus DNAs

The *polyhedrin* and *enhancin 3* genes were amplified by PCR from AcMNPV C6 and TnGV DNAs, respectively, to obtain fragments of 1073 bp and 2738 bp (data not shown). Recombinant AcMNPV viruses that included the *ph* gene (BacPh), and the *ph* and *en3* genes (BacPhEn3) were constructed using Bac-to-Bac technology. Correct insertion of these genes was confirmed by restriction endonuclease (REN)

analysis (Figure 1a) and sequencing analysis following PCR amplification of adjacent regions using the M13-Fw and M13-Rv primers (Figure 1b).

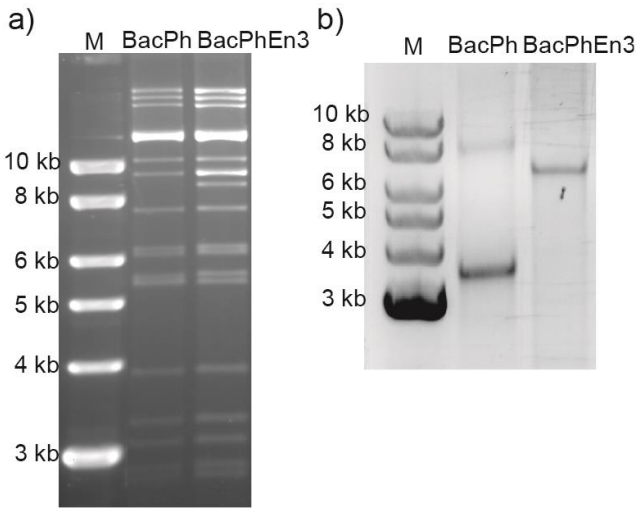

Figure 1. (**a**) *Pst*I restriction endonuclease profiles of recombinant bacmids BacPh and BacPhEn3. The strong band above the 10 Kb marker corresponds to the Bac-to-Bac helper plasmid. (**b**) Inverted image of PCR products obtained after amplification of recombinant DNAs using M13-Fw and M13-Rv primers. The image was inverted for improved clarity.

PCR amplicons of ~3.5 kb and ~6.5 kb for BacPh and BacPhEn3, respectively, were in line with the expected sizes of 3633 and 6371 bp obtained after adding 2560 bp to each of the PCR products following the Bac-to-Bac protocol (ThermoFisher Scientific, Waltham, MA, USA). In fact, the *en3* was a 2738 bp fragment, so the fragment obtained with BacPhEn3 DNA was ~3 kb larger in the gel. Sequence analysis confirmed that the *ph* gene in BacPh and BacPhEn3 recombinants and the *en3* in the BacPhEn3 recombinant were inserted correctly after the *p10* promoter and *ph* promoter, respectively. DNA was then purified and transfected into Sf9 cells for the production of viral OBs.

3.2. Transfection of Sf9 cells with recombinant BacPh and BacPhEn3 virus DNAs

At five days after transfection, most of the cells infected with BacPolhEn3 had OBs in the cell nuclei at levels similar to those of the control virus (BacPolh) and AcMNPV C6. Recombinant viruses produced normal OBs observable by optical

microscopy (Figure 2a). The shape and size of OBs were indistinguishable among the three viruses (Figure 2a) and the ODV content (number of infectious units) of samples comprising 5 × 10^8 OBs did not differ significantly among these viruses (ANOVA, $F_{2,6}$ = 0.919; p = 0.449) (Figure 2b).

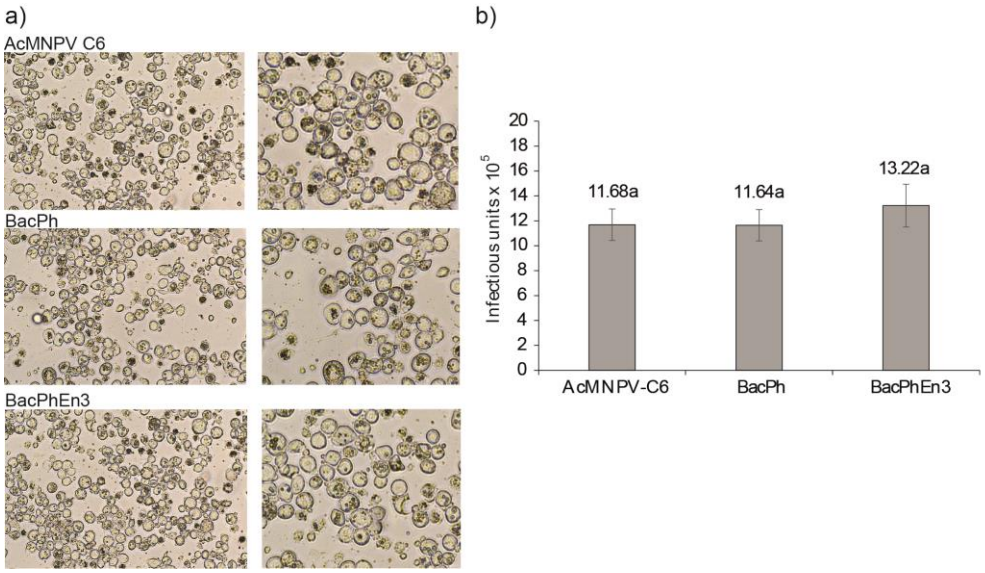

Figure 2. a) Sf9 cells infected with AcMNPV-C6 BVs at 10 m.o.i., and Sf9 cells transfected with recombinant BacPh and BacPhEn3. The images were taken five days after infection. Images of cells presenting normal presence and abundance of OBs in cell nuclei. **b**) Results of end-point dilution assay of the ODVs released from samples of 5 x 10^8 OBs obtained from AcMNPV-C6 OBs and cell pellets of infected BacPh and BacPhEn3 cells showing similar numbers of infectious units (ANOVA, p > 0.05). Values above columns indicate means. Error bars indicate standard deviation.

Examination of restriction endonuclease profiles and PCR analysis of recombinant OBs was performed to corroborate their identity in comparison with the original samples. Infected cells were recovered and lysed to obtain viral OBs, and DNA extraction was performed on these OBs. REN analyses showed that viral DNA profiles from BacPolh and BacPolhEn3 transfections (Figure 3a) were the same as those of the original inocula (shown in Figure 1a). M13-Fw and M13-Rv primers produced a PCR product of ~3.5 kb and ~6.5 kb for BacPh and BacPhEn3, respectively, which corresponded with the expected sizes (Figure 1b). Finally, sequence analysis confirmed the presence of these genes in the correct position, and that the recombinant viruses were correctly designed and constructed (data not shown).

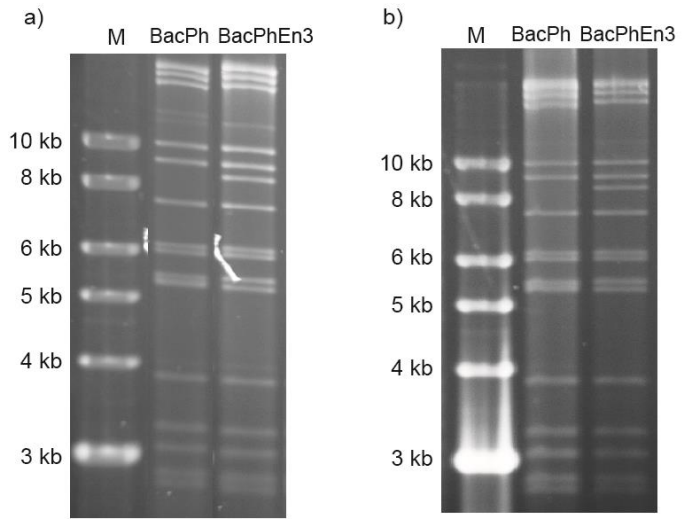

Figure 3. Restriction endonuclease profiles with *Pst*I of **a**) cell pellets transfected with recombinant bacmids BacPh and BacPhEn3, and **b**) OBs purified from larvae after injection of transfection supernatant (BVs) in *Spodoptera exigua* fifth instars. The molecular size marker, M, was smart ladder (Stratagene).

3.3. Enhancin 3 is solubilized in the cell medium

The supernatant containing BVs was used to inject *S. exigua* fifth instar larvae to produce large quantities of OBs. Injection of BacPh and BacPhEn3 BVs resulted in a similar prevalence of larvae mortality (range 86–95%). Dead larvae were collected five to seven days after infection and showed the typical signs of lethal polyhedrosis disease. OBs were purified and were checked by REN and PCR analysis. REN treatment of BacPh and BacPhEn3 OBs resulted in the characteristic profiles of each virus (Figure 3b), identical to those of the original inoculated viruses (shown in Figure 1a). Sequence analysis of PCR products confirmed that sequences were identical to those of the original inocula. Therefore, the BacPh and BacPhEn3 OBs produced in larvae were confirmed to be those of the recombinant viruses and were used for bioassays.

3.4. Enhancin 3 is solubilized in the cell medium

To detect En3, a sample separation protocol was followed (Figure 4a), and an aliquot of each sample was used to perform SDS-PAGE (Figure 4b).

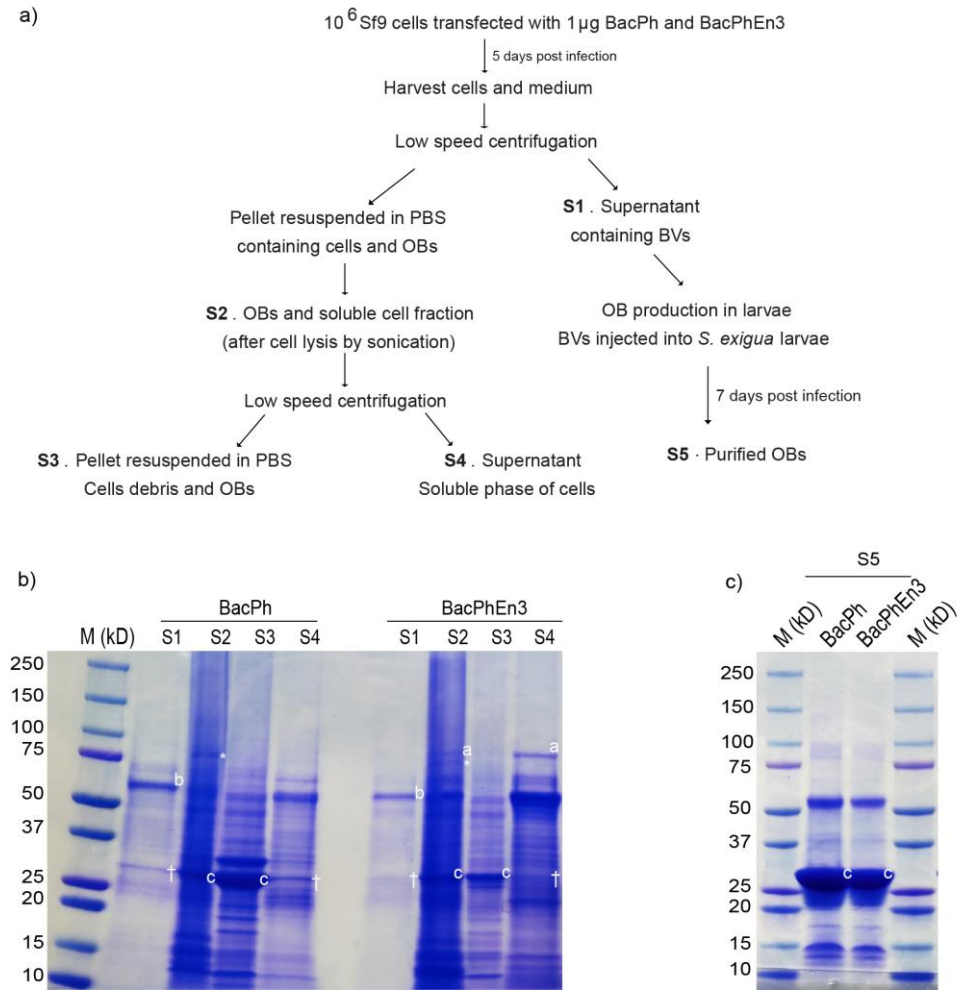

Figure 4. a) Sample extraction protocol for virus-infected cells and OBs resulting in five types of samples (S1–S5). **b**) SDS-PAGE of samples S1–S4 obtained from cell culture. **c**) SDS-PAGE of sample S5 purified from virus-killed larvae shown in **a**). For each sample, a 20 µL sample was subjected to SDS-PAGE. Letters on gel image indicate "**a**" the En3 protein in S2 and S4 in BacPhEn3, "**b**" GP64 in S1 for both BacPh and BacPhEn3 and "**c"** the polyhedrin in S2, S3 and S5, for both BacPh and BacPhEn3. Signs on gel images indicate "†" a band of similar to polyhedrin and "*" the band immediately below the En3 protein in S2 in both BacPh and BacPhEn3.

The En3 protein was present in Sf9 cells transfected with the recombinant BacPhEn3 but was not present in BacPh transfected cells. The SDS-PAGE analysis revealed that the BV fraction (S1) presented a major band of 56.8 kDa, corresponding to the GP64 protein (indicated by the letter “a” in both BacPh and BacPhEn3; Figure 4b), and lacked the band corresponding to En3. Cell pellets resulting from infection with BacPhEn3 showed a band of ~100 kDa that corresponded in size to En3 (indicated by the letter “c” in S2 in BacPhEn3; Figure 4b), which was absent in the S2 sample of BacPh. A band of similar size to En3 was present in the S2 sample of BacPh, but this had a lower molecular weight and also appeared below the En3 band in S2 of BacPhEn3 (indicated with an asterisk in S2 in both BacPh and BacPhEn3). Moreover, in S4 of BacPhEn3, only the upper band (labeled “a”) was present, corresponding to En3, which co-migrated with En3 in S2 of BacPhEn3 (also labeled “a”), rather than the lower band (labeled “*”) in S2 of BacPhEn3.

Cell pellets were lysed by sonication and the pellet, and the supernatant obtained after low-speed centrifugation showed the band of ~100 kDa that corresponded in size to En3 (indicated with the letter “c” in S4 in BacPhEn3; Figure 4b). As a control, cells infected with BacPh did not show the band corresponding to En3 in the supernatant of cell lysate (S4 sample in BacPh; Figure 4b). The purified OBs from lysed cells (S3) did not show the band corresponding to En3, indicating that this protein was not present in the OBs or ODVs at detectable quantities (S3 sample in BacPhEn3; Figure 4b). A band corresponding to En3 was not observed in the OBs purified from larvae and was therefore also not present in the ODVs at detectable levels (S5 sample; Figure 4c).

Finally, the polyhedrin protein of 27 kDa was present in cell pellets and OBs produced in larvae infected by BacPh and BacPhEn3 (S2 and S3; Figure 4b and S5; Figure 4c), indicating the correct production of this OB protein, as expected. In the S4 samples of BacPh and BacPhEn3, a faint band of similar size to Polh was present (indicated with a cross in S4) but was clearly less intense than in S2 and S3. To separate the OBs (S3) and the soluble fraction of the cells (S4), a low-speed centrifugation was performed; therefore, the soluble fraction (S4) may have contained traces of OBs. It is also possible that this band could be another cellular protein, as it also appeared in the S1 sample (BV fraction) in both BacPh and BacPhEn3.

These results clearly indicated that En3 was produced but was not incorporated into OBs or enveloped within ODVs, but instead accumulated in the cell medium as a soluble entity.

3.5. Biological activity of BacPhEn3 OBs produced in cell culture and in larvae

Discriminating concentration insect bioassays were performed with the OBs and cell lysate from cell culture (equivalent to S2 in Figure 4a) and with OBs purified from larvae (equivalent to S5 in Figure 4a). In second instars (Figure 5a), a significant interaction effect was detected in virus × origin (cells or larvae) of OBs, presumably due to the presence of En3 in the cell lysate of BacPhEn3-infected cells ($F_{1,32}$ = 5.460; p = 0.026). The origin of the inoculum (cells vs. larvae) also interacted significantly with inoculum concentration ($F_{1,32}$ = 6.982; p = 0.013). The main effects of virus ($F_{1,32}$ = 0.882; p = 0.355) and inoculum origin ($F_{1,32}$ = 0.058; p = 0.812) were not significant, whereas mortality increased significantly with OB concentration, as expected ($F_{1,32}$ = 214.71; p < 0.001).

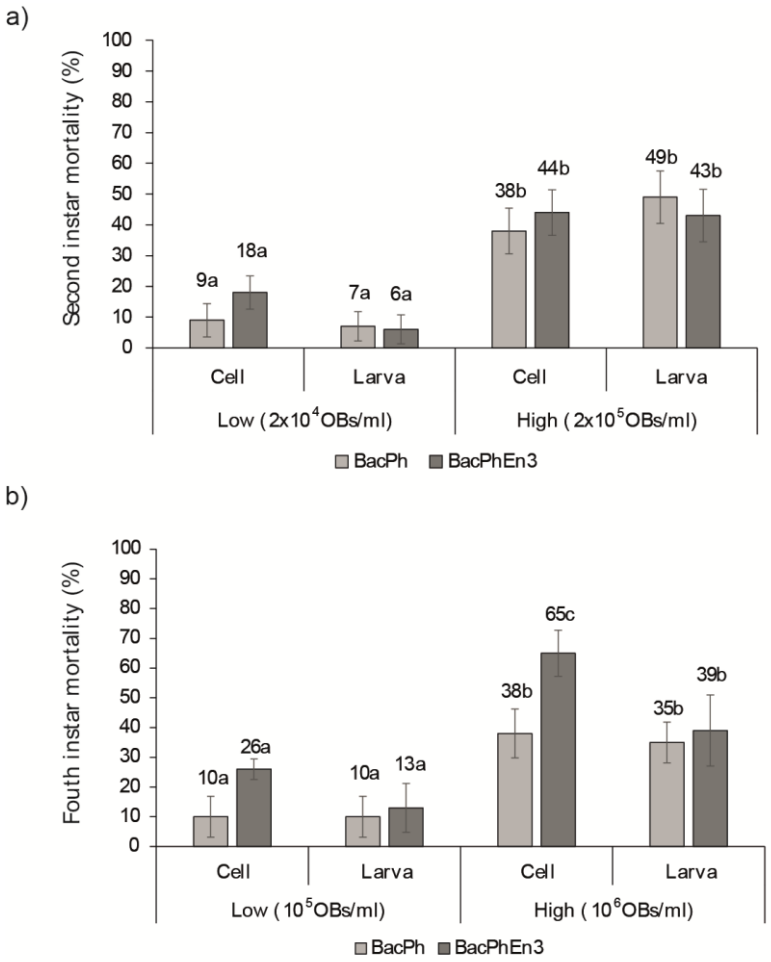

Figure 4. Percentage of larval mortality induced by BacPh and BacPhEn3 OBs produced in cell culture (OBs + cell lysate) and OBs purified from larvae in **a**) second instars and **b**) fourth instars of *Spodoptera exigua*. Second instars were inoculated with 2×10^4 OBs/mL or 2×10^5 OBs/mL and fourth instars with 10^5 OBs/mL or 10^6 OBs/mL. Values above columns indicate mean percentage of mortality. Values followed by identical letters did not differ significantly (ANOVA, Tukey $p < 0.05$). Error bars indicate SD.

The results differed markedly in fourth instars compared to second instar larvae (Figure 5b). The main effects of virus ($F_{1,32} = 21.20$; $p < 0.001$), inoculum origin ($F_{1,32} = 15.43$; $p < 0.001$) and inoculum concentration ($F_{1,32} = 122.54$; $p < 0.001$) significantly affected larval mortality. There was also a significant interaction of virus × origin of OBs ($F_{1,32} = 11.572$; $p = 0.002$), due to the presence of Eh3 in the cell lysate of BacPhEn3 OBs + cell lysate, which resulted in the highest prevalence of mortality in the high inoculum concentration (65%, Figure 5b), and its presumed absence in BacPhEn3 OBs purified from larvae (39% mortality). The tendency was

the same in the low inoculum concentration, although the mortality induced by BacPh OBs and BacPhEn3 OBs did not differ significantly when inoculated in mixtures with infected cell lysate (Tukey, $p > 0.05$). None of the other interaction terms were significant ($p > 0.05$).

As the discriminating concentration assays revealed that BacPhEn3 OBs + cell lysate resulted in high mortality in fourth instar larvae, a series of concentration-mortality response bioassays was performed. Specifically, the LC_{50} values and relative potencies of OBs were estimated for both recombinants obtained from cell culture and larvae based on the prevalence of virus-induced mortality observed across a range of five OB concentrations (Table 2).

Table 2. OB pathogenicity (LC_{50}) in *Spodoptera exigua* second and fourth instars inoculated with BacPh and BacPhEn3 OBs + cell lysate from infected Sf9 cells and BacPh and BacPhEn3 OBs purified from larvae following injection with BacPh and BacPhEn3 BVs.

Instar	Virus	Slope ± SE	LC_{50} (OBs/mL)	95% Conf. Interval		Relative	95% Conf. Interval	
				Lower	Upper	Potency	Lower	Upper
Second instar	**OBs and cell lysate from Sf9 cells**							
	BacPh	0.306 ± 0.078	1.33×10^5	4.30×10^4	8.75×10^5	1		
	BacPhEn3	0.573 ± 0.084	4.51×10^4	2.37×10^4	8.73×10^4	2.95	1.03	11.40
	OBs purified from larvae following injection with BVs							
	BacPh	0.555 ± 0.088	1.15×10^5	5.93×10^4	2.58×10^5	1.16	0.27	5.03
	BacPhEn3	0.488 ± 0.093	2.03×10^5	8.73×10^4	7.52×10^5	0.65	0.13	3.33
Fourth instar	**OBs and cell lysate from Sf9 cells**							
	BacPh	0.847 ± 0.126	1.10×10^6	6.15×10^5	2.84×10^6	1	-	-
	BacPhEn3	1.019 ± 0.119	2.34×10^5	1.66×10^5	3.53×10^5	4.69	2.63	10.59
	OBs purified from larvae following injection with BVs							
	BacPh	0.830 ± 0.110	1.09×10^6	6.38×10^5	2.14×10^6	1.01	0.40	2.07
	BacPhEn3	0.756 ± 0.106	1.13×10^6	6.32×10^5	2.37×10^6	0.97	0.37	2.02

Relative potency values were calculated with respect to the BacPh OBs + cell lysate (control) treatment in both instars. Goodness-of-fit tests were non-significant for second ($\chi^2 = 6.82$; df = 3; $p = 0.078$) and fourth ($\chi^2 = 2.85$; df = 3; $p = 0.416$) instars.

In second instar larvae, the BacPhEn3 OBs + cell lysate were 2.95-fold more pathogenic than control BacPh OBs + cell lysate, presumably due to the presence of En3 in the lysate fraction. In contrast, BacPh and BacPhEn3 OBs purified from larvae were as pathogenic as the control virus with relative potency values of 1.16 and 0.65,

respectively, with broadly overlapping 95% confidence limits, reflecting the absence of En3 in these samples.

The enhancement of infection of BacPhEn3 OBs + cell lysate was more marked in fourth instar larvae. The BacPhEn3 OBs + cell lysate was 4.69-fold more pathogenic than control BacPh OBs + cell lysate, whereas BacPh OBs and BacPhEn3 OBs purified from larvae were as pathogenic as the control BacPh OBs + cell lysate with a relative potency of 1.01 and 0.97, respectively. These findings confirmed that the lysate of BacPhEn3-infected cells was responsible for the potentiation of AcMNPV infection.

4. DISCUSSION

The localization of a granulovirus enhancin in NPV-infected Sf9 cells and its enhancing activity was investigated by constructing an AcMNPV recombinant that expressed the enhancin 3 (*en3*) gene of TnGV. The enhancin protein was localized to the soluble fraction of the cells and was not present in NPV OBs (or ODVs) in detectable quantities. Following cell lysis, the enhancing factor present in the lysate was capable of potentiating NPV infection by ~3-fold in second instars and ~4.7-fold in the fourth instar larvae of *S. exigua*.

These findings are consistent with the purification of enhancin from *T. ni* cells infected with recombinant AcMNPV that expressed the TnGV En3 reported by Lepore et al. (1996). In contrast, Hayakawa et al. (2000) used intact virus infected Sf9 cells as inoculum. These cells were infected with a polyhedrin-negative AcMNPV recombinant virus that expressed the En3 of TnGV, so that larvae in the bioassay ingested mixtures of wild-type AcMNPV OBs and cells infected by the recombinant virus that likely contained the enhancin protein. In another study, recombinant virus expressing the TnGV enhancin (En3) was subjected to three rounds of passage in cell culture prior to insect bioassay. The resulting OBs were observed to have modest levels (two-fold) of potentiation activity in a diet contamination bioassay, although the purification steps of the OBs were not described (Del Rincón-Castro and Ibarra, 2005). We assume that the OB preparations used by these authors included traces

of enhancin from the host cells, which resulted in the modest levels of potentiation observed.

In the present study, we demonstrated that the lysate of infected cells contained the potentiation factor rather than the recombinant OBs or ODVs. The BacPhEn3 OBs purified from infected larvae by filtration and differential centrifugation were as pathogenic as control BacPh OBs purified from larvae or BacPh OBs inoculated in mixtures with cell lysate. Potentiation activity was only observed for BacPhEn3 OBs when in mixtures with the lysate of BacPhEn3-infected cells. The prevalence of virus-induced mortality could have been influenced by the presence of non-occluded virions in cells. If so, such entities would be present in both control (BacPh) and BacPhEn3 treatments involving cell lysate. In addition, the BV and OB titers obtained in infected cells were similar for the two viruses, suggesting similar titers of non-occluded virions in both BacPh and BacPhEn3 treatments. Therefore, we can conclude that the potentiation effect was due to the presence of En3 in the lysate of BacPhEn3-infected cells.

When purifying OBs from virus-killed insects, once the infected larvae had liquefied, they were homogenized and filtered to eliminate debris. The resulting suspension was subjected to various rounds of low-speed centrifugation to pellet the OBs, whereas the supernatant containing traces of cell debris and cellular proteins was discarded to finally obtain semi-purified OBs (Durantel et al., 1998; Simón et al., 2004). Therefore, any enhancing factor was eliminated or markedly reduced in concentration during OB purification from larvae. It is possible that bioassay results could have been influenced by the presence of non-occluded virions in cell lysates. However, if present, such entities would be present in similar quantities in both control (BacPh) and BacPhEn3 treatments involving cell lysate, so we are confident that the effects attributed to the presence of enhancin are real and not an inadvertent effect of the presence of virions that had not been incorporated into OBs.

The enhancing activity demonstrated in this study (~3-fold and ~4.7-fold in second and fourth instars, respectively) was broadly in line with that reported by other authors (Del Rincón-Castro and Ibarra, 2005; Lepore et al., 1996), although Hayakawa et al. (2000) reported higher potentiation of AcMNPV and SeMNPV infection in *S. exigua* third instars, for the reasons mentioned above. In other systems,

a recombinant AcMNPV expressing the enhancing-like protein of AgseGV was fivefold more pathogenic than the wild-type OBs (Zhang et al., 2012). Using a fusion technology, the AgseGV enhancin and GP37 were embedded independently into two different recombinants, and the recombinant bacmid OBs were three- and fivefold more pathogenic than wild-type OBs, respectively (Yang et al., 2017). The GP37 is homologous to the glycoprotein fusolin present in entomopoxvirus spindles, the N-terminal of which binds to chitin and markedly enhances infection by entomopoxviruses and nucleopolyhedroviruses (Takemoto et al., 2008). Similarly, using the same technology, both AgseGV enhancin and GP37 were embedded in the same recombinant OBs that were 3.9-fold and 7.4-fold more pathogenic to second and fourth instar larvae than wild-type OBs, respectively (Lei et al., 2019).

We observed a clear instar-dependent effect in the potentiation activity of BacPhEn3 cell lysate. Enhancin potentiation tends to be greater in late instars that are normally more resistant to baculovirus infection than their younger counterparts (Lei et al., 2019; Wang and Granados, 2001; Wang et al., 1994). The lepidopteran PM contains pores that vary in size among the different species and across larval instars (Wang and Granados, 2001). In early instars, the PM is generally less tightly constructed and more permeable, whereas in later instars, the PM is well-formed and has lower porosity (Erlandson et al., 2019; Peng et al., 1999; Wang and Granados, 2001). Therefore, PM disruption by these enzymes is greater in late instars and the potentiation effect more marked as late instar larvae are usually more resistant to baculovirus infection.

Enhancin orthologues are present in a few group II alphabaculoviruses (Bischoff and Slavicek, 1997; Li et al., 2003; Popham et al., 2001; Slavicek and Popham, 2005; Thiem, 2009). These proteins are present within ODVs in association with nucleocapsids, where they appear to facilitate access to the midgut cells (Slavicek and Popham, 2005; Toprak et al., 2012). They also target IIM, with the mucins being common targets for both GV and NPV enhancins (Shi et al., 2004; Toprak et al., 2012). Indeed, GV and NPV enhancins are similar but differ in several aspects. They share up to 39% identity, range in size from 758 to 848 aa, and contain a conserved zinc-binding domain that is characteristic of metalloproteases (Erlandson et al., 2019; Slavicek et al., 2012). However, NPV enhancins contain a potential transmembrane

domain followed by a series of basic amino acid residues at their carboxyl terminus (Figure S1) (Erlandson et al., 2019). The presence of this transmembrane domain allows them act as fusains targeted at midgut cells (Erlandson et al., 2019). The absence of the transmembrane domain in granulovirus enhancins would prevent the envelopment of TnGV En3 in the AcMNPV ODV membrane. In contrast, NPV enhancins are able to insert into the ODV membrane of AcMNPV. For example, the Mamestra configurata NPV enhancin was found to be a component of the ODVs of a recombinant AcMNPV expressing this protein (Toprak et al., 2012), and the recombinant OBs were over fourfold more pathogenic than wild-type OBs (Li et al., 2003).

As GV and NPV enhancins clearly have different functions, the incorporation of a GV-type enhancin within alphabaculovirus OBs would appear challenging. This was recently achieved by fusion of the enhancin gene with the polyhedrin gene (Je et al., 2003; Lei et al., 2019; Yang et al., 2017). The application of gene fusion technology has the potential to create a novel protein expression system and an efficient virus-based system for insecticide production in countries that allow the use of genetically modified organisms in agriculture (Sun, 2015). However, the use of recombinant viruses in agriculture is currently prohibited in many countries including those of the European Union (Directive 2001/18/CE).

5. CONCLUSIONS

In the present study, a granulovirus enhancin produced by an AcMNPV recombinant accumulated within infected cells and was shown to be present in the soluble fraction of the cells following lysis. The soluble fraction was responsible for potentiation of AcMNPV infection in *S. exigua* larvae and was more effective in fourth instars than in second instar larvae. The production of solubilized enhancins using a baculovirus-based expression systems could be used to improve the efficacy of biological insecticides against lepidopteran pests.

6. APPENDIX A

To select a suitable enhancin for the construction of the recombinant virus, a literature survey was carried out to identify the betabaculovirus enhancins with the highest potentiation activity.

The potentiation activity of betabaculovirus enhancins has been extensively studied. In contrast, NPV enhancins are present only in a few NPVs and their location within the ODVs (Slavicek and Popham, 2005; Toprak et al., 2012) hinders their purification and has restricted their study as synergists of viral infection.

In an early study, a factor present in Pseudaletia unipuncta (Psun) GV was observed to increase the infectivity of PsunNPV OBs in mixed infections (Tanada, 2012). Later, Granados et al. demonstrated that the enhancin from TnGV degraded major glycoproteins within the PM (Derksen et al., 1988, Lepore et al., 1996). To date, the enhancins of *Agrotis segetum* (Agse), *Epinotia aporema* (Epap), *Helicoverpa armigera* (Hear), *Spodoptera frugiperda* (Sf), *Trichoplusia ni* (Tn), *Pseudaletia unipuncta* (Psun) and *Xestia c-nigrum* (Xecn) GVs have been demonstrated to enhance baculovirus infections (Biedma et al., 2015; Gijzen et al., 1995; Guo et al., 2007; Jeyarani and Karuppuchamy, 2010; Lei et al., 2019; Tanada et al., 1975). Enhancins from TnGV have been the most frequent subject of study. Enhancement activity has been demonstrated by different approaches, particularly the use of enhancins purified from GV granules (Gallo et al., 1991; Lepore et al., 1996; Wang and Granados, 1997; Wang et al., 1994). They have been assayed in mixtures with NPV OBs (Hayakawa et al., 2000; Biedma et al., 2015; Jeyarani and Karuppuchamy, 2010) by constructing recombinant baculoviruses (Del Rincón-Castro and Ibarra, 2005; Hayakawa et al., 2000; Lei et al., 2019; Yang et al., 2017) or transgenic plants expressing enhancin genes (Cao et al., 2002; Mori et al., 2006).

Among the different enhancin species, the TnGV enhancins produced the highest enhancement of baculovirus infections (Gallo et al., 1991; Wang et al., 1994). Reports of enhanced infection by AcMNPV in mixtures with purified TnGV enhancins vary from a 60 to 63% increase in *T. ni* (Gallo et al., 1991; Lepore et al., 1996) to 82% in *Pseudaletia unipuncta* (Gallo et al., 1991; Tanada et al., 1975). Using recombinant

viruses expressing the TnGV enhancin, the pathogenicity of NPV OBs increased 39-fold for *S. exigua* larvae inoculated with AcMNPV, or 10-fold for larvae inoculated with SeMNPV (Hayakawa et al., 2000). Larvae with a diet based on transgenic plants or mixtures of OBs and transgenic tobacco expressing enhancin showed increased susceptibility to infection (Cao et al., 2002; Mori et al., 2006). The delta-endotoxins of *Bacillus thuringiensis* were also more effective when formulated with TnGV enhancin (Granados et al., 2001; Guo et al., 2019). Taken together, these findings led us to select TnGV enhancins for our study. These proteins were also subjected to a molecular analysis (Appendix B) to identify the presence of active domains.

7. APPENDIX B

A protein analysis was performed to identify the presence of active domains in TnGV enhancins. Three enhancins have been identified in the genome (Bivian-Hernández et al., 2017), and a conserved domain search was performed with NBCI batch Web CD-Search Tool (Marchler-Bauer and Bryant, 2004) in order to select one to construct the recombinant AcMNPV. De novo discovery of motifs was carried out using MEME suite (Bailey et al., 2009). TMHMM Server v. 2.0 was used to predict transmembrane helices in proteins (Sonnhammer et al., 1998; Krogh et al., 2001). Protein properties were identified using the Protein Molecular Weight Calculator available online: http://www.sciencegateway.org/tools/proteinmw.htm (19/05/2021). Enhancin protein sequences included in the analysis are available in the NCBI GenBank database under accession numbers: YP_002268112.1 (AgipNPV_En), YP_009513096.1 (AgseGV_En), AAP29820.1 (CfMNPV_En), AAG33872.1 (ChfuGV_En AAG33872.1), AFP66947.1 (DekiNPV_En), YP_001649133.1 (HearGV_En1), YP_001649134.1 (HearGV_En2), YP_001649135.1 (HearGV_En3), YP_001649146.1 (HearGV_En4), NP_047702.1 (LdMNPV_En1), NP_047797.1 (LdMNPV_En2), AAM09197.1 (MacoNPV-A_En), NP_689263.1 (Maco_NPV-B_En), ACU46624.1 (MbMNPV_PEn), YP_009249965.1 (MolaGV_En3), YP_009249970.1 (MolaGV_En4), YP_009666739.1 (MyunNPV_En), YP_009345853.1 (MyunGV_En2), AUA60360.1 (OpbrNPV_En), YP_009049908.1 (PespNPV_En), YP_003422496.1 (PsunGV_En1), YP_003422498.1 (PsunGV_En3), YP_003422509.1 (PsunGV_En4), AXS01151.1 (SfGV_En1), YP_009506217.1 (TnGV_En1), YP_009506219.1 (TnGV_En3), YP_009506228.1 (TnGV_En4),

NP_059298.1 (XecnGV_Orf150), NP_059300.1 (XecnGV_Orf152), NP_059302.1 (XecnGV_Orf154) and NP_059314.1 (XecnGV_Orf166).

The en1, en3, and en4 enhancin genes from TnGV have open reading frames of 2487 bp, 2706 bp, and 2574 bp, and encode proteins of 828, 901, and 857 amino acids with a molecular weight of 91, 100, and 95 kDa, respectively. Pairwise identities among the three enhancins were less than 32%. Differences in amino acids sequences altered the predicted conserved domain sequences and the predicted transmembrane helices present in each enhancin protein (Figure S1). Within En3, the zinc metallopeptidase motif (HEXXHX(8,28)E) (indicated by light-green residues in Figure S1) is present in the Peptidase_M60 Superfamily domain (marked in a green box in Figure B1), as in almost all metalloproteases (Slavicek , 2012). In contrast, En1 and En4 lack this motif. The Mucin_bdg super family domain, putative mucin, or carbohydrate binding domain for the substrates of enhancin (inside blue box in Figure S1) and other similar metallopeptidases are present in En3 and En4 proteins and closely related enhancin proteins. Interestingly, the Peptidase_M60 Superfamily domain and transmembrane helices (marked in red text in Figure S1) are absent in En1, En3, and En4 proteins and closely related enhancins. In contrast to NPV enhancins, GV enhancins do not possess the transmembrane helix domains necessary for insertion in the ODV envelope (Figure S1).

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CHAPTER IV

Lab-scale production of enhancin En3 from Trichoplusia ni granulovirus and its synergistic activity with other baculoviruses

ABSTRACT

Enhancins are metalloproteinases that disrupt the peritrophic matrix favoring baculovirus infections. An enhancin factor (En3) from Trichoplusia ni granulvoirus (TnGV) expressed using a bacmid system was found in large quantities in the cell soluble fraction. The goal of the present study was to evaluate the feasibility of mass-producing it *in vitro* and *in vivo*, with the aim of incorporating the cell soluble fraction as a synergist in baculovirus formulations. The *in vivo* system in *S. exigua* or *T. ni* larvae was more productive than that *in vitro* in Sf9 cells. *In vivo*, larvae were infected with the recombinant virus and collected before they liquefied. Larvae were then incubated at 26ºC for 24 h to allow for cell lysis and liquefaction and the subsequent release of En3 into the soluble fraction. The soluble fraction was then purified, and special care was taken to eliminate infectious recombinants. This soluble fraction, without any other further purification, at 100 ng/□l, was capable of potentiating baculovirus infectivity in homologous hosts by 15-fold and by 4-fold in heterologous hosts. The soluble fraction of larvae infected with recombinant bacmid expressing the En3 represents a promising additive to potentiate baculovirus formulations.

This chapter

1. INTRODUCTION

Compounds with protease activity can alter the structural integrity of the insect peritrophic matrix (PM) (Brandt et al., 1978), increasing its permeability to pathogens. Baculovirus enhancins are metalloproteinases that significantly increase viral infection in lepidopteran insects by targeting the insect intestinal mucin (IIM), the major mucinous protein in the PM of lepidopteran larvae (Erlandson et al., 2019; Wang and Granados, 2001). Enhancin genes are more prevalent in lepidopteran-infecting granuloviruses (GV, genus *Betabaculovirus*), where they are present in the occlusion body (OB) matrix (Lepore et al., 1996; Erlandson et al., 2019). The virus-enhancing activity of betabaculovirus enhancins has been well-documented (Erlandson et al., 2019, Gallo et al., 1991; Gizjen et al., 1995; Lei et al., 2019). They are present in high amounts in the granules, up to 5% (Erlandson et al., 2019; Gallo et al., 1991; Roelvink et al., 1995), allowing their purification in large quantities. Alkaline soluble proteins of GV capsules and other microbial pathogens have also been postulated as promising additives for virus formulations (Biedma et al., 2015; Granados et al., 2001; Guo et al., 2019; Mukawa and Goto, 2007; Wang and Granados, 2001). However, before their inclusion in formulated products, mass production of the granules followed by costly purification of the enhancin by gel filtration chromatography, are needed.

Engineered baculoviruses expressing betabaculovirus enhancins with enhanced infectivity are effective biocontrol agents (Lei et al., 2020, Yang et al., 2017). Employing bacmid technology, recombinant NPVs expressing enhancins have been constructed with up to 5 to 20-fold higher pathogenicity than parental viruses (Del Rincón-Castro and Ibarra, 2015; Hayakawa et al., 2010; Lei et al., 2019; Lepore et al., 1996; Yang et al., 2017). Recently, a novel strategy to incorporate enhancins into alphabaculovirus OBs was postulated as a highly efficient virus-based system for insecticide production. By fusing the enhancin gene to a C-terminal section of the polyhedrin gene, the enhancins are occluded within the OBs making them more effective against lepidopteran larvae (Lei et al., 2019; Yang et al., 2017). However, following this technology the protein of interest might be embedded in the OBs making its purification more difficult. Meanwhile, the release of recombinant

viruses to the environment is currently prohibited in many countries, including those of the European Union (Directive 2001/18/CE).

Recently, it was found that a recombinant AcMNPV expressing an unmodified enhancin 3 from Trichoplusia ni granulvoirus (TnGV) non-fused to polyhedrin accumulated the enhancing factor in the cell soluble fraction (En-CSF) (Ricarte-Bermejo et al., 2021). What is the same, the lysate of infected cells contained the potentiation factor rather than the recombinant OBs or ODVs. The En-CSF was responsible for the potentiation effect of AcMNPV infection in *Spodoptera exigua* larvae due to the presence of En3 in the lysate of infected cells (Ricarte-Bermejo et al., 2021). The production of solubilized enhancins using baculovirus-based expression systems could be used in formulated products to improve the efficacy of baculovirus and other biological insecticides against lepidopteran pests.

In the present study, the production and purification of Trichoplusia ni granulovirus (TnGV) En3 was carried out using a baculovirus expression vector both *in vitro* and *in vivo*. The goal was to evaluate the feasibility of its mass-production with the aim of incorporating the En-CSF as a synergist in baculovirus formulations. Different parameters were studied in both systems such as harvesting time and insect species while the viability of both systems was evaluated. The En-CSF was used in combination with different baculovirus species in homologous and heterologous hosts to evaluate its enhancing activity.

2. MATERIAL AND METHODS

2.1. Insect, cells and viruses

Larvae of *Trichoplusia ni*, *S. exigua, Spodoptera littoralis, Spodoptera frugiperda, Mamestra brassicae, Chrysodeixis includes and Chrysodeixis chalcites*, all of them from the order Lepidoptera and the family Noctuidae, were obtained from laboratory colonies maintained on semisynthetic diet (Greene et al., 1976) at 25 °C. Sf9 cells (ThermoFisher Scientific, Waltham, MA, USA) were maintained in TC100 medium (Lonza Bioscience, Cologne, Germany) supplemented with 10% fetal calf serum (Lonza Bioscience, Cologne, Germany) at 28 °C (King and Possee, 1992). The BacPhEn3, a polyhedrin positive bacmid that expressed the En3 from TnGv was

constructed in a previous study (Ricarte-Bermejo et al., 2021). The BacPh, which did not contain the En3 was used as a control virus in the production system. The Spodoptera exigua NPV (SeMNPV), S. littoralis NPV (SpliNPV) and the broader host range Autographa californica NPV (AcMNPV-C6) were obtained from the baculovirus collection of the Microbial Bioinsecticides group at the Public University of Navarre.

2.2. En-CSF production and purification in Sf9 cells

2.2.1. Optimal harvesting moment

To determine the highest pick of En3 production and accumulation within infected cells, the optimal harvesting moment was determined. Four 75 cm^2 flasks containing 10^7 cells were infected at 10 m.o.i., and 3, 4, 5, and 6-days post infection, the medium and the cells containing the OBs were recovered and centrifuged at 2400× g for 5 min. The BV-containing supernatant was stored at 4ºC, and the pellet,containing the cells and the OBs, was resuspended in 500 µl PBS. The infected cells were lysed by sonication in an ultrasonic bath (Selecta Master, JP Selecta, Abrera, Spain) for 10 min at 100 W. Finally, 20 µl samples of the lysate from each harvesting time point were subjected to SDS-PAGE to estimate the time of highest enhancin production. The protein concentration at each harvesting time point was measured by the Bradford assay (Bradford, 1976) (Bio-Rad, Berkeley, CA, USA) using bovine serum (BSA) as standard. The experiment was repeated three times. The protein concentration data was analysed by an ANOVA test (IBM SPSS Statistics 27 Software, Windows) as data were normally distributed. Means were compared by Tukey test. Finally, the BV stock solution was tittered by plaque assay (King and Possee, 1992; O'Reilly et al., 1994).

2.2.2. En-CSF purification

The En-CSF was purified to eliminate contaminants, particularly occluded and non-occluded viruses that might have an influence on larval mortality. Five 75 cm^2 flasks containing 10^7 cells were infected at 10 m.o.i. and, four days post-infection, the cells and the medium were recovered. Cells and OBs were pelleted and the supernatant containing the BVs was kept a 4ºC. The pellet was then resuspended in 500 µl PBS. The infected cells were lysed by sonication in an ultrasonic bath (Selecta

Master, JP Selecta, Abrera, Spain) for 10 min at 100 W. The soluble fraction was obtained by centrifugation at 5900× g for 5 min. The pellet (cells debris and occluded OBs) was discarded and the supernatant (En-CSF) was then filtered through 0.45 μm and 0.2 μm filters (Minisart®, Sartorius, Germany) to eliminate the remainder OBs and non-occluded OBs (ODVs).

To determine if En3 remained soluble and passed throughout the filters, 20 μl of unfiltered supernatant and 20 μl of each filtrate was subjected to a SDS-PAGE. Samples were mixed with 2x sample buffer (Bio-Rad), boiled at 100 °C for 5 min, and then subjected to electrophoresis as previously described (Laemmli, 1970), using Criterion TGX™ 4-20% Precast Gel (BIO-RAD). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad) and then distained in 30% ethanol and 10% acetic acid. Protein was quantified by the Bradford assay (Bradford, 1976) (Bio-Rad), using bovine serum albumin (BSA) as a standard, for each repetition. Data were analysed using IBM SPSS Statistics 27 Software, Windows.

Finally, mortality induced by unpurified and purified (0.45 μm and 0.2 μm) En-CSF was determined by infecting fourth instar *S. exigua* larvae with the droplet feeding method (Hughes and Wood, 1976). Larvae were starved for 8-12 h at 25ºC and then allowed to drink from an aqueous suspension containing the En-CSF at 200 ng/μl and 10% sucrose plus 0.001% (w/v) Fluorella Blue. Larvae that ingested the suspension within 10 min were transferred individually to the wells of a 28-well dish with a piece of semi-synthetic formaldehyde-free diet (Greene et al., 1981). Bioassays were performed three times using groups of 28 larvae per virus concentration and 28 mock-infected control larvae. Larvae were reared at 26 ± 1 ºC and mortality was recorded daily until insects had either died or pupated. Virus-induced mortality results were subjected to ANOVA analysis using the SPSS Statistics 27 package (IBM Corp., Armonk, New York). Means were compared by Tukey test.

2.3. En-CSF production and purification in larvae

2.3.1. Insect species

To select the insect species more suitable for the production of the soluble enhancing factor, late instar larvae of different species were infected with BacPhEn3. Twenty-eight L_6 *C. chalcites*, L_6 *C. includens*, L_6 *M. brassicae*, L_5 *S. exigua*, L_6 *S. frugiperda*, L_6 *S. littoralis*, and L_6 *T. ni*, were injected with 5 μl BV stock diluted 1:1000 ($1x10^7$ BVs) (Ricarte-Bermejo et al., 2021). This BV stock was the same as that produced after transfection with BacPhEn3 DNA to avoid serial rounds of infection that could lead to the excision of the gene of interest and to a rapid decrease in En3 production (Pijlman et al., 2003). Larvae were inspected daily. The experiment was repeated three times. Mortality data was analysed using ANOVA test (IBM SPSS Statistics 27 Software, Windows). Means were compared by Tukey test.

2.3.2. Production and purification in T. ni *and* S. exigua *larvae*

Ten *T. ni* and *S. exigua* late instars were infected as mentioned above and mortality was inspected daily. When larvae showed clear symptoms of infection and before they liquefied, they were collected and transferred collectively to a 15 ml Falcon vials. Once all the larvae died, the tube was incubated at 26 ± 1 ºC for 24h to allow for the liquefaction of all tissues and cell lysis, and the release of the En3-containing soluble fraction. Larvae were homogenized using a 10 ml pipette in 10 ml milli-Q water. The homogenate was then filtered through a piece of muslin cloth to eliminate larva debri. Thereafter, OBs were separated from the supernatant (ie. the soluble fraction of infected cells) by centrifugation at 5900× g for 5 min. The pellet containing the OBs was discarded and the supernatant was passed through a 0,2 μm filter and transferred to new 15 ml tubes. The protein concentration for each species was measured by Bradford (Bradford, 1976). Twenty μl of the En-CSF produced by each species were subjected to SDS-PAGE to detect enhancin production in different hosts. The experiment was repeated three times for each species. Concentration data was analysed using t-student test (IBM SPSS Statistics 27 Software, Windows).

2.4. Bioassays

2.4.1. Synergistic effects of En-CSF *produced both* in vitro *and* in vivo

The potentiation effect of the solubilized enhancins produced both *in vitro* and *in vivo* was compared in a concentration-mortality assay. SeMNPV OBs were mixed with the same quantity of the En-CSF, 200 ng/μl, and the mixture was used to infect fourth instar *S. exigua*. Larvae were allowed to drink from aqueous suspensions containing two viral concentrations, low ($5x10^3$ OBs/ml) and high ($5x10^4$ OBs/ml), the purified enhancin at 200ng/μl and 10% sucrose plus 0.001% (w/v) Fluorella Blue (Hughes and Wood, 1981). Larvae that ingested the suspension within 10 min were transferred individually to the wells of a 28-well dish with a piece of semi-synthetic formaldehyde-free diet (Greene et al., 1976). Bioassays were performed five times using groups of 28 larvae per virus concentration and 28 mock-infected control larvae. Larvae were reared at 26 ± 1 ºC and mortality was recorded daily until insects had either died or pupated. Virus-induced mortality was checked daily and mortality data was analysed by ANOVA test (IBM SPSS Statistics 27 Software, Windows). Means were compared by Tukey test.

2.4.2. Minimum concentration of En-CSF *enhancing OB infections*

To determine the minimum concentration of En-CSF that induced the highest effect on baculovirus pathogenicity, *S. exigua* fourth instars were infected by the droplet feeding method with their homologous virus and the En-CSF at different concentrations. The protein concentrations were determined by Bradford (Bradford, 1976). Larvae were allowed to drink from aqueous suspensions containing 10^5 OBs/ml of SeMNPV, the En-CSF at 10 ng/μl, 50 ng/μl, 100 ng/μl, 250 ng/μl and 500ng/μl and 10% sucrose plus 0.001% (w/v) Fluorella Blue. Larvae that ingested the suspension were transferred individually to 28-well dishes with a piece of semi-synthetic diet (Greene et al., 1976). Bioassays were performed five times using groups of 28 larvae per virus concentration and 28 mock-infected control larvae. Larvae were reared at 26 ± 1 ºC and mortality was recorded daily until insects had either died or pupated. Virus-induced mortality was checked daily and mortality data

was analysed by ANOVA test (IBM SPSS Statistics 27 Software, Windows). Means were compared by Tukey test.

2.4.3. Potentiation induced by the En-CSF

The minimal soluble fraction concentration was assayed in pathogenicity assays using different baculovirus species in homologous and heterologous fourth instar larvae. SeMNPV and SpliNPV were assayed in their homologous hosts and AcMNPV in the heterologous *S. exigua*. Firstly, SeMNPV, SpliNPV and AcMNPV OB concentrations were quantified in a Neubauer hemocytometer.

The increased pathogenicity induced by the enhancer substance was measured in terms of 50% lethal concentration (LC_{50}) and compared with that produced by OBs alone (control). Bioassays were performed following the droplet feeding method (Hughes and Wood, 1981) on fourth instars. Groups of larvae were starved for 8-12 h at 25 ºC and then allowed to drink from an aqueous suspension containing 100 ng/µl of the En-CSF, 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and one of five OB concentrations. For SeMNPV the concentrations were $2x10^7$, $4x10^6$, $8x10^5$, $1.6x10^5$ and $3.2x10^4$ OBs/ml for virus alone and $4x10^6$, $8x10^5$, $1.6x10^5$, $3.2x10^4$ and $6.4x10^3$ when the En-CSF was added. For SpliNPV, $2.5x10^6$, $5x10^5$, $1x10^5$, $2x10^4$ and $4x10^3$ OBs/ml were applied when using only the virus and $5x10^5$, $1x10^5$, $2x10^4$ and $4x10^3$ $8x10^2$ in combination with the En-CSF. Finally, AcMNPV alone was used at $1x10^8$, $2x10^7$, $4x10^6$, $8x10^5$ and $1.6x10^5$ OBs/ml and at $1x10^7$, $2x10^6$, $4x10^5$, $8x10^4$ and $1.6x10^4$ OBs/ml in combination with the En-CSF. These concentrations were previously determined to kill between 95% and 5% of the experimental insects for each of the species tested. Similarly, larvae that ingested the suspension within 10 min were transferred individually 28-well dishes and mortality was checked daily. Bioassays were performed three times using groups of 28 larvae per virus concentration and 28 mock-infected control larvae. Virus-induced mortality results were subjected to probit analysis using the Polo-Plus program (Le Ora software, 1984).

3. RESULTS

3.1. En-CSF production and purification in Sf9 cells

3.1.1. Harvesting moment

The En3 protein was detected in the soluble fraction of the cells infected with BacPhEn3 (Figure 1a), and not in BacPh transfected cells. The highest quantity of soluble protein was obtained four days after transfection with the BacPhEn3 ($F_{(3,8)}$=69.8, P<0.005). At day 4, the protein production peaked, 2409 ng/μl (±156 SD), and was significantly higher than those obtained at day 3 (2068 ng/μl ±41), 5 (2150 ng/μl ± 59) and 6 (1421 ng//μl ±28) post-transfection (Figure 1b). The production obtained at days 3 and 5 was similar (Tukey test, p>0.05). In contrast, at day 6 after transfection, the protein production dropped significantly (Tukey test, p<0.05). For comparison, the protein quantity produced by the BacPh at day 4 post-infection reached 316 ng/μl + 105.

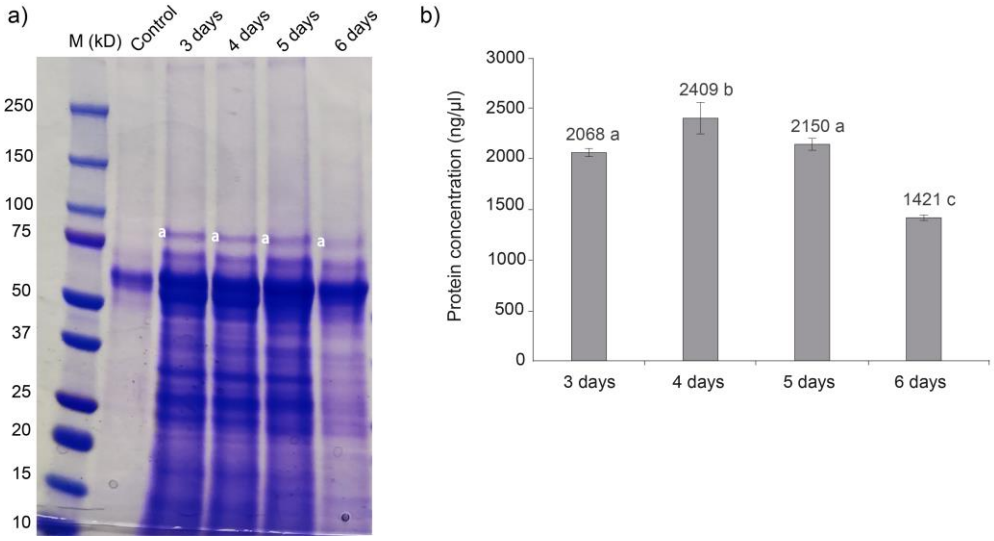

Figure 1. a) SDS-PAGE of soluble fraction haversted at different time points (Lines 3-6). As a negative control, the soluble fraction of the cells infected with BacPh was included in the SDS-PAGE (Lane 2). M (kD) represents the protein marker Precision Plus Protein Standards (Bio-Rad, Berkeley, CA, USA). The letter "a" indicates the En3 protein band; b) Protein concentration values obatined in the soluble fraction of the cells infected with BacPhEn3 harvested at different time points. Values above bars indicate means and those followed by identical letters did not differ significantly (Tukey test, p<0.05). Error bars indicate the standard deviation.

3.1.2. En-CSF purification

To eliminate the potential presence of infectious viruses, the En-CSF obtained from each flask was sieved through 0.45 μm and 0.2 μm filters. The enhancin remained in the soluble fraction after purification with the two filters (Figure 2a). The Ph protein, of 30 kDa (Figure 2a), was present in the infected cells after lysis, but was not evident in unfiltered cell soluble fractions and in those filtered through 0.45 μm and 0.2 μm filters, indicating that most of the occluded viruses remained in the pellet after centrifugation of cell lysate.

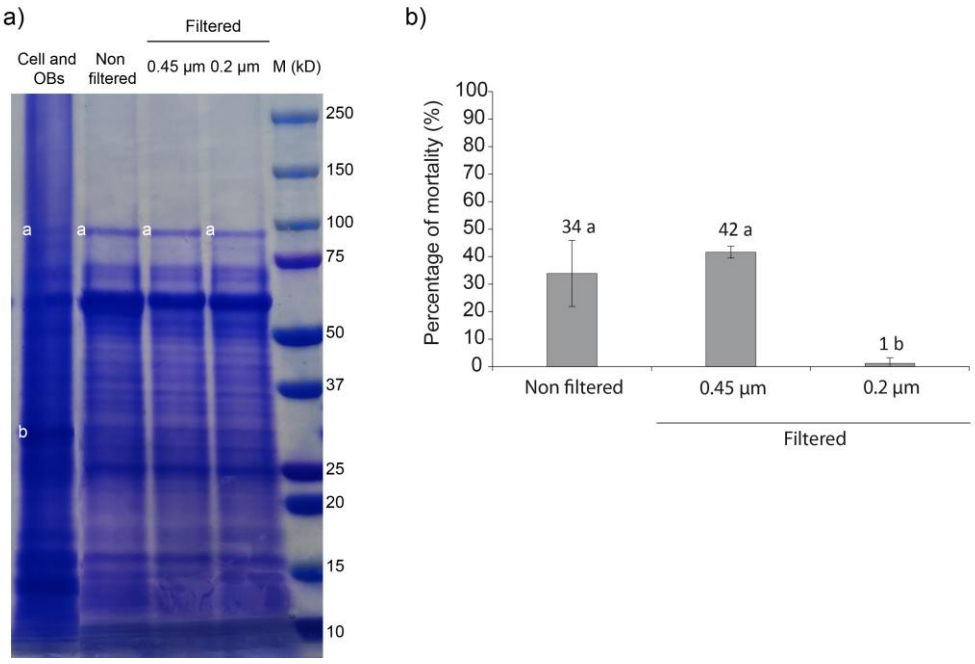

Figure 2. a) SDS-PAGE of the soluble fraction of cells infected with BacPhEn3 and purified by filtering. Lane 1 contains the cells infected with BacPhEn3 after lysis; lane 2 the nonfiltered soluble fraction of cells after low centrifugation; lane 3 the soluble fraction filtered through 0.45 μm; and lane 4 the filtration through 0.2 μm. M (kD) stands for the protein marker Precision Plus Protein Standards (Bio-Rad, Berkeley, CA, USA). Letter "a" means En3 protein and "b" polyhedrin. b) Percent mortality obtained after infection of fourth instar larvae with 200 ng/μl soluble fractions before and after filtering through 0.45 μm and 0.2 μm pores. Values above bars indicate means and those followed by identical letters did not differ significantly (Tukey test, $p<0.05$). Error bars indicate the standard deviation.

To confirm the elimination of occluded and non-occluded viruses that might influence the bioassays, *S. exigua* fourth instar larvae were infected with the unfiltered and filtered fractions (Figure 2b). The unfiltered lysate and the lysate filtered

through 0.45 μm induced a higher mortality than the 0.2 μm filtrate (ANOVA $F_{(2,7)}$=17.16, P<0.005). The unfiltered fraction and the 0.45 μm filtrate produced 33.9% ± 12% (medium ± SD) and 41.6% ± 2.2% mortality, respectively, with no significant differences between them (Tukey test, p>0.05). In contrast, the 0.2 μm filtrate yielded 1.2% ± 2.1% mortality (only one larvae in only one of the three replicates died). The 0.45 μm filter did not eliminate non-occluded viruses, while that of 0.2 μm eliminates the infectious viruses that might magnify synergism assays. However, using the *in vitro* system the final volume obtained after purification of the En-CSF was as low as ≈500 μl/75 cm^2 flask.

3.2. En-CSF production and purification in larvae

3.2.1. Insect species

To select the most appropriate insect species for the production of solubilized enhancing, the latest instars of different species were infected with BacPhEn3 BVs. The mortality obtained in the different species varied significantly ($F_{(3,8)}$ = 12.83, p = 0.002). *T. ni* and *S. exigua* mortality were as high as 97% ± 5.8% (medium ± SD) and 93% ± 5.7%, respectively, while that of *C. includens* was 87% ± 6.0%. No significant differences were obtained between them (Tukey test, p>0.05). However, *C. includens* larvae were more fragile than *S. exigua* and *T. ni* and more than 20% of larvae died during the injection and manipulation. Moreover, *S. exigua* and *T. ni* larvae took 4-5 days to die, while *C. includes* 7 days. For these reasons, *C. includens* larvae were not considered appropriate for enhancin production. In *S. frugiperda,* BacPhEn3 induced lower mortality 60% ± 10% (Tukey test, p<0.05) while the latest instars of *C. chalcites*, *S. littoralis, M. brassicae* did not die. In sum, *T. ni* and *S. exigua* larva were selected to produce En3 for synergism assays.

3.2.2. Production of the En-CSF in S. exigua *and* T. ni *larvae*

Ten larvae of *T. ni* and *S. exigua* were infected with BacPhEn3 and collected jointly before liquefying. Dead larvae were homogenized in 10 ml miliQ and the En-CSF was purified by filtering first throughout muslin to eliminate larva remains and then by centrifugation to pellet the OBs. The supernatant (ie. the soluble fraction) was then passed through a 0.2 μm filter to eliminate potential infectious recombinants.

The filtrate retained the En3 in the soluble fraction (Figure 3a) and the centrifugation eliminated the apparent occluded viruses (Figure 3a). Ten ml of solubilized enhancin was obtained for each species and replicate. Both species produced the same quantity of solubilized proteins (t= 0.408; d.f.= 4; p= 0.589), being 2093 ng/µl ± 259 (mean + SD) in *S. exigua* ,and 1998 ng/µl ± 308 in *T. ni*. Both species were suitable for the production of En3. However, for subsequent assays the En-CSF produced in *S. exigua* was used for practical reasons (at the time of the assays, the *S. exigua* colony was more robust and easier to handle than the *T. ni* population).

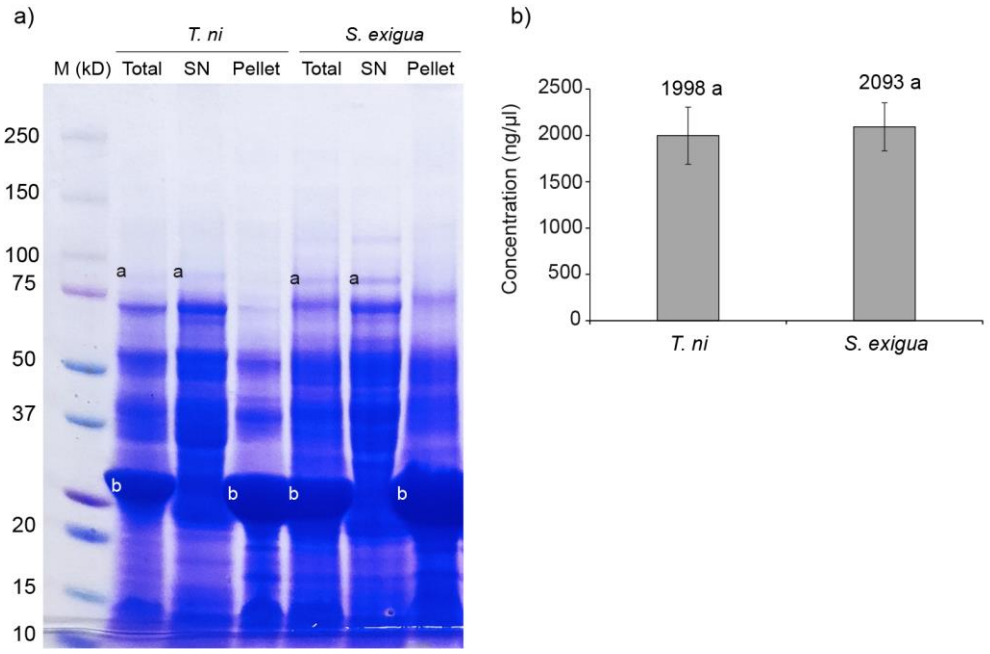

Figure. 3. a) SDS-PAGE of the cell soluble fraction (supernatant, SN) and the OBs (Pellet) obtained in *S. exigua* and *T. ni* larvae. Lane 1, M (kD), stands for protein marker Precision Plus Protein Standards (Bio-Rad, Berkeley, CA, USA). Lanes 2 and 5 contained a pool of larval homogenate without purification; lanes 3 and 6 the soluble cell fraction after centrifugation and filtering; and lanes 4 and 7 the pellets with the OBs and cell debris obtained after centrifugation for each species. Letter “a” indicates the En3 protein and “b” polyhedrin. B) Protein quantity obtained in the soluble fractions of *S. exigua* and *T. ni* larvae was quantified by Bradford. Values above bars indicate means and those followed by identical letters did not differ significantly (Tukey test, p<0.05). Error bars indicate the standard deviation.

3.3. Synergism assays

3.3.1. Enhancement activity of En-CSF produced in vitro *and* in vivo

The increase in activity induced by the En-CSF produced from both *in vitro* and *in vivo* systems was compared in *S. exigua* fourth instars using its homologue virus. The En-CSF obtained from cultured cells or larvae enhanced SeMNPV infectivity similarly. At low concentrations, the *in vitro* and *in vivo* cell soluble fractions produced significantly higher mortality ($F_{(3,12)}$=13.54, P<0.005), of 46.6% ± 14.2% (mean ± SD) and 49.3% ± 9.8%, respectively, than the control virus (22.7% ± 1.8%). Both systems potentiated SeMNPV pathogenicity equally (Tukey test, p>0.05). Similarly, at high SeMNPV OB concentrations, the inclusion of En-CSF induced higher mortality, 84.3% ± 4.5% from *in vitro*-produced soluble fractions and 85.7% ± 4.2% from that obtained *in vivo*, while mortality by the control virus was 73.7% ± 1.5% ($F_{(3,8)}$=5.74, P<0.05). Both production systems enhanced virus activity in a similar way (Tukey test, p>0.05). Although the two systems were valid for the production of soluble enhancing fractions, *in vivo* production yielded much higher En3 volumes and this system was selected for further experimentation.

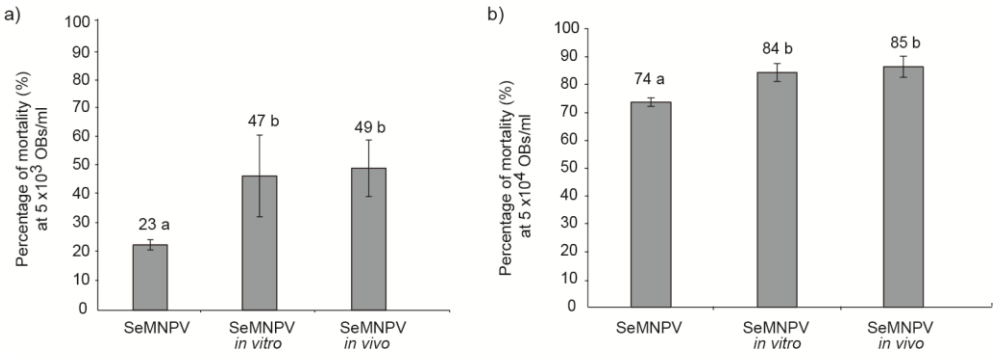

Figure 4. Enhancement of SeMNPV activity in *S. exigua* fourth instar larvae by the addition of 200 ng/µl enhancer substance obtained *in vitro* and *in vivo*. Larvae were infected with a) $5x10^3$ OBs/ml and b) $5x10^4$ OBs/ml. Mortality data was compared with that obtained with the virus alone. Values above bars indicate means and those followed by identical letters did not differ significantly (HSD Tukey test, p<0.05). Error bars indicate the standard error of the mean.

3.3.2. Minimum concentration of En-CSF that enhanced OB infections

There was a clear concentration-dependent effect, as the more quantity of En-CSF, the highest the synergistic effect on SeMNPV mortality (ANOVA $F_{(5,12)}$=4.8, P<0.05) (Figure 5). At low concentrations of En-CSF, there was not a potentiation effect and the mortality was similar as in the control treatment, of 39% ± 3.0% (mean ± SD) for the control virus and of 46% ± 9.5% and 48% ± 9.1% for treatments with 10 and 50 ng/µl soluble fractions, respectively(Tukey test, p>0.05). At higher concentrations, the addition of En-CSF clearly increased SeMNPV mortality (Tukey test, p<0.05). One hundred ng/µl, 250 ng/µl and 500 ng/µl produced 55% ± 5.1%, 56% ± 1.0% and 60% ± 3.6% mortality, respectively, with no significant differences between them (Tukey test, p>0.05). Therefore, the minimum concentration of the enhancer substance fraction that produced a significant viral potentiation was 100 ng/µl.

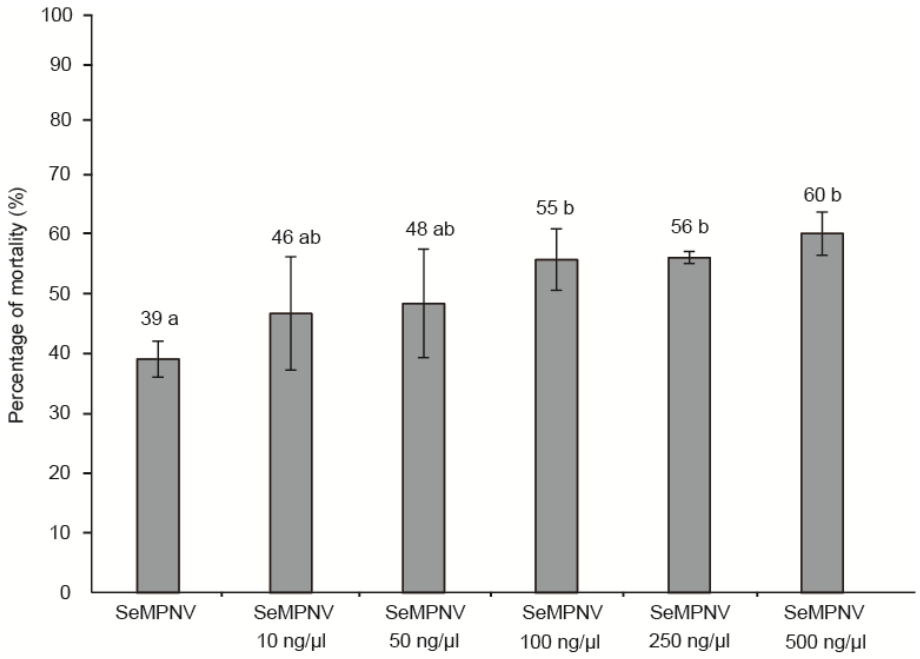

Figure 5. Enhancement of SeMNPV activity in *S. exigua* fourth instar larvae by the addition of different concentrations of En-CSF obtained from the *S. exigua in vitro* system. Larvae were infected with 10^4 OBs/ml and increasing concentrations of En-CSF. The mortality data were compared with those obtained with the virus alone. Values above bars indicate means and those followed by identical letters did not differ significantly (HSD Tukey test, p<0.05). Error bars indicate the standard error of the mean.

3.3. Potentiation induced by the *in vivo* En-CSF

Pathogenicity assays were performed with the En-CSF at 100 ng/µl. The CL_{50}s and the relative potencies were calculated for SeMNPV and SpliNPV in their homologous fourth instar hosts, and also on AcMNPV in the heterologous host *S. exigua* (Table 1). The addition of 100 ng/µl of soluble fraction decreased the LC_{50} of SeMNPV by 15-fold, from 1.15 $x10^5$ to 7.7 $x10^3$ OBs/ml. In the SpliNPV-*S. littoralis* system, the En-CSF reduced the LC_{50} from 5.1 $x10^5$ OBs/ml to 4.4 $x10^4$, which means a 11.6-fold reduction of the effective concentration. In contrast, the enhancement of AcMNPV activity over the heterologous host employed, *S. exigua,* was lower, of only 4.2-fold from 4.8$x10^5$ OBs/ml to 1.1$x10^5$ OBs/ml. The enhancement effect was clearly more intense in the homologous systems.

Table 1. LC_{50} and relative potencies of SeMNPV and SpliNPV on their homologous fourth instar hosts, and AcMNPV in the heterologous *S. exigua* fourth instar in presence or absence of the En-CSF at 100 ng/µl.

Treatments	Slope ± SE	CL_{50} (OBs/ml)	Fiducial limits 95%		Relative Potency	Fiducial limits 95%	
			Lower	Upper		Lower	Upper
SeMNPV	0.628 ± 0.073	1.15 $x10^5$	6.86 $x10^4$	2.24 $x10^5$	1	-	-
SeMNPV+ En3	0.455 ± 0.069	7.68 $x10^3$	3.40 $x10^3$	1.49 $x10^4$	15.01	5.96	37.75
SpliNPV	0.593 ± 0.091	5.11 $x10^5$	2.26 $x10^5$	1.71 $x10^6$	1	-	-
SpliNPV+ En3	0.711 ± 0.094	4.39 $x10^4$	1.87 $x10^4$	1.34 $x10^5$	11.64	4.50	30.15
AcMNPV	0.961 ± 0.111	4.78 $x10^5$	3.17 $x10^5$	6.79 $x10^5$	1	-	-
AcMNPV+ En3	0.395 ± 0.071	1.13 $x10^5$	2.51 $x10^4$	3.03 $x10^5$	4.22	1.55	11.52

Relative potency values were calculated with respect to the parental virus. Goodness-of-fit tests were non-significant for SeMNPV (χ^2 = 2.99; df = 1; p = 0.084) and for SpliNPV (χ^2 = 0.81; df = 1; p = 0.367) and significant for AcMNPV (χ^2 = 20.37; df = 1; p <0.001).

4. DISCUSSION

Enhancins are metalloproteinases that degrade the insect intestinal mucin, the major protein component of the peritrophic matrix (PM), increasing permeability to insect pathogens. Recently, it was found that an enhancin expressed in a baculovirus expression system localized in the soluble fraction of the cells and, upon cell lysis, it potentiated NPV infection (Ricarte-Bermejo et al., 2021). In the present study, the soluble fraction of cells expressing En3 was produced and purified with the aim of using it in combination with baculovirus OBs. The soluble fraction of lysed cells contained also occluded and non-occluded infectious viruses, which clearly influenced the infectivity of the inoculum (Ricarte-Bermejo et al., 2021). The use of 0.2 μm filters eliminated the infectious viruses from the cell soluble fraction, and retained the enhancing factor solubilized in the medium. This filtrate can be used directly as an adjuvant in formulated products.

The En-CSF was produced both *in vitro* and *in vivo* to determine different production parameters. In the *in vitro* system, the highest En3 yields were obtained at day four after infection. Thereafter, protein production dropped quickly. Bac to bac expression system are based on the expression of foreign genes under the p10 and polh promoters, which are active during the very late phase of baculovirus infection (Luckow et al., 1993; Smith et al., 1983; van Oers et al., 2015; Vlak et al., 1990). During this period proteases are also released to the medium, which might result in proteolytic degradation of the recombinant protein (Brondyk, 2009). The increase in activity obtained with both systems was similar, although the volume of soluble fraction obtained in the *in vivo* system was mucha higher. As much as 10 ml of En3 soluble fraction were obtained by infecting 10 *S. exigua* or *T. ni* larvae. In contrast only 500 μl per 75cm^2 flask were obtained in cultured cells, which makes this system inviable. *S. exigua* and *T. ni* larvae were the most susceptible species, and therefore, the most appropriate for the production of En3 *in vivo*. These two insect species are very permissive to AcMNPV , while *S. frugiperda, C. includens*, *S. littoralis* or *M. bassicae* are semipermissive (Cohen et al., 2009; Xu et al., 2012). The soluble fractions produced both *in vitro* or *in vivo* in *S. exigua* or *T. ni* larvae enhanced baculovirus infection at a similar rate. In relation to costs, *in vitro* production is more expensive (Inceoglu et al., 2001) and leads to a rapid decrease in protein production

due to passage effect (Pijlman et al., 2003). In sum, considering all aspects, the *in vivo* system was selected for the massive production of a soluble fraction with synergistic activity for baculovirus biopesticides.

The enhancing factor present in the soluble fraction potentiated AcMNPV pathogenicity in the heterologous *S. exigua* by 4.2-fold, consistent with previous results (Del Rincón-Castro et al., 2005; Hayakawa et al., 2010; Lepore et al., 1996; Ricarte-Bermejo et al., 2021). However, the synergistic effect was more intense in the homologous systems. SeMNPV and SlMNPV were15 and 11fold more pathogenic against *S. exigua* and S. littoralis, respectively, when the synergistic factor was added, The specificity of baculoviruses is determined by their ability to enter the cells of susceptible hosts initially, and then to replicate and produce new infectious virus particles (Rohrmann, 2019; Simón et al., 2004). Throughout these processes, the PM is not the only barrier to a productive infection and several mechanisms operate in conferring host cell specificity at different steps. Therefore, the enhancing effect is higher in homologous systems, in which host permisivity exists and in which disruption of the PM matrix increases primary infection rates of highly specific viruses.

Larvae are markedly less susceptible to infection as they age (Asser-Kaiser et al., 2011; Kouassi et al., 2009). This stage-related resistance to infection increases steadily with larval body weight in some species (Myers et al., 2011). In many cases, the physiological basis for this process remains uncertain. Larvae are able to rid themselves of primary infection by sloughing off infected gut cells during the molt (Washburn et al., 1998; Rohrmann, 2019). The PM is also a key barrier against infection by baculoviruses, and larvae can resist infection by increasing the thickness of the PM (Levy et al., 2007; Plymale et al., 2008; Wang and Granados, 2001; Zhu et al., 2007). Susceptibility to viral infection decreased through successive instars as the PM became progressively less permeable (Lehane, 1997). In this regard, enhancins are effective at increasing the insecticidal efficacy of OBs (Okuno et al., 2003; Guo et al., 2007; Toprak et al., 2012; Wang and Granados, 2001). When the En3-containing soluble fraction was added to the OBs, the effective viral concentration against L_4 larvae was reduced to a level similar to that for L_2 larvae (Murillo et al., 2003). This means that a single concentration may be able to control

a range of instars. Under field conditions, effective crop protection is favoured when all pest instars can be controlled following a single application of a viral insecticide, since in natural populations, overlap of larval generations may be common. Due to the lower susceptibility of late instars to baculovirus infections (Bernal et al., 2014), the enhancer substance produced in the present study has attracted interest to control late instars, the principal cause of feeding damage in crops.

In the present study, we demonstrated that the soluble fraction of infected insects purified through 0.2 μm filters without any other purification is sufficient to enhance baculovirus infections. This system reduces the production costs, compared with the alkaline soluble proteins of GV capsules (Granados et al., 2001; Wang and Granados, 2001), whose GVs OBs have to be firstly massively produced and thereafter the soluble proteins purified through gel filtration chromatography.

5. CONCLUSIONS

In the present study, an enhancer substance is produced and used in combination with baculoviruses. The soluble fraction of the cells without gel chromatography purification is capable of potentiating baculovirus infectivity in homologous hosts up to 15-fold and up to 4-fold in heterologous systems. Using this enhancing substance, a single baculovirus concentration could effectively control a range of different instars. Moreover, only fifty larvae would be necessary to produce the quantity of En3 necessary to treat 1 Ha.

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7. ACKNOWLEDGMENTS

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CHAPTER V

General discussion

This study arises from the need to solve one of the major drawbacks of baculovirus-based insecticides: the high costs associated with their mass production (Grzywacz et al., 1998; Gupta et al., 2007; Hunter-Fujita, 1998). To make them commercially competitive with chemical insecticides, coadjuvants that allow reductions in the amount of active compounds in bioinsecticide formulations are a must. As such, peritrophic matrix (PM) disrupting agents, which increase PM permeability to microbial pathogens, have been postulated as promising additives in baculovirus products (Guo et al., 2007; Wang and Granados, 2001; Zhu et al., 2007). In the present thesis, the potential application of an enhancin as a synergist in baculovirus-based insecticides was evaluated.

Firstly, an extensive revision of enhancins along with other PM disrupting agents, such as optical brighteners and chitinases that increase baculovirus and other microbial pathogens infectivity, was performed with the aim of selecting the most appropriate enhancin for its production as a synergistic product. Among the different disruption agents, optical brighteners produced by far the highest enhancement of baculovirus infection (García-González and Genersch, 2013; Wang and Granados, 2001; Zhu et al., 2007). These, are broad-range chemical compounds with extremely high chiting-binding properties that completely disrupt PM structure (Wang and Granados, 2001). However, their chemical origin renders them unacceptable for biological control products. In contrast, naturally occurring enhancins and chitinases, have also protease activity to degrade the PM and facilitate the initial virus infection

(Berini et al., 2016; Biedma et al., 2015; Erlandson, et al., 2019; Rao et al., 2004, Slavicek, 2012; Wang and Granados, 2001). However, they have been poorly investigated as enhancers of baculovirus infection (Shapiro et al., 1987).

Baculovirus enhancins found in granuloviruses and nucleopolyhedroviruses both degrade the PM (Toprak et al., 2012; Wang and Granados, 1997), but they target the PM differently. Granuloviruses release a large amount of enhancins into the larval midgut, which then degrade the PM in a non-targeted random manner (Slavicek, 2012). In contrast, NPVs utilize enhancins located in ODV envelopes to "tunnel" through the PM to gain access to larval midgut cells, and the enhancin activity is located on the ODV-PM interaction site (Hoover et al., 2010; Slavicek and Popham, 2005). They degrade the insect insecticidal mucin (IIM) in a more subtle way than the prominent degradation caused by betabaculovirus enhancins (Slavicek, 2012). This led us to select a betabaculovirus enhacin for the construction of the expression vector. Among the different betabaculovirus enhancin species, TnGV enhancins have been by far the most studied and shown the highest enhancement of infection (Gallo et al., 1991; Wang et al., 1994). Purified TnGV enhancins have demonstrated to enhance AcMNPV infection by around 60% in *T. ni* larvae (Gallo et al., 1991; Lepore et al., 1996) and 82% in *Pseudaletia unipuncta* (Tanada et al., 1975; Wang et al., 1994). Similarly, recombinant viruses expressing the TnGV enhancin augmented the pathogenicity of NPV OBs by 39-fold for *S. exigua* larvae inoculated with AcMNPV, or by 10-fold for larvae inoculated with SeMNPV (Hayakawa et al., 2010). Three enhancins have been identified in the genome of TnGV (Bivian-Hernández et al., 2012). One of them, En3, contains the zinc metallopeptidase motif (HEXXHX(8,28)E), as in almost all metalloproteases (Slavicek , 2012). In contrast, En1 and En4 lack this motif. The Mucin_bdg super family domain is present in En3 and En4 proteins and closely related enhancin proteins. Both domains are key in conferring the structural and functional properties of enhancins (Ishimwe et al., 2015). Therefore, the En3 was selected as an enhancing factor for its expression and production in a bacmid system. Interestingly, En3, as all sequenced GV enhancins, lack the transmembrane domain that locate the NPV enhancins in ODV membranes.

One question concerning the recombinant En3 expressed by an NPV is whether the recombinant enzyme is occluded within OBs, as in GVs, or integrated

into the ODV envelope, as occurs in NPVs. This has a clear implication in the lab-scale production of enhancins using baculovirus expression systems. Determining the location of the enhancin is crucial to design a proper production system. As GV and NPV enhancins clearly have different functions, the incorporation of a GV-type enhancin within alphabaculovirus OBs would appear challenging. Moreover, the incorporation of a betabaculovirus enhancin into alphabaculovirus OBs has only been achieved by fusing the *enhancin* gene to a C-terminal section of the *polyhedrin* gene (Lei et al., 2019; Yang et al., 2017). However, it was unclear whether unmodified betabaculoviruses not fused with polyhedrin incorporated the enhancin to NPV OBs. The application of gene fusion technology has the potential to create efficient virus-based systems for insecticide production (Lei et al., 2019, Yang et al., 2017). However, the use of recombinant viruses in agriculture is currently prohibited in the European Union (Directive 2001/18/CE). Meanwhile, the enhancin occlusion within the OBs hinders its purification and use as a synergistic agent.

To overcome this problem, a polyhedrin positive recombinant AcMNPV expressing TnGV En3 was constructed. The recombinant En3 was successfully produced, and the En3 accumulated in infected cells, specifically in the soluble fraction of the cells following lysis, and not in the OBs or ODVs (Ricarte-Bermejo et al., 2021). The absence of a transmembrane domain in En3 seemed to preclude the attachment to ODV envelopes, and the fact that NPV OBs do not embed enhancins made their occlusion in OBs unlikely. Indeed, enhancins were present in large quantities in the cell medium. These findings were consistent with the purification of enhancin from *T. ni* cells infected with a recombinant AcMNPV expressing TnGV En3 (Lepore et al., 1996). What is more, this soluble fraction increased the infectivity of NPVs (Ricarte-Bermejo et al., 2021). These results allow the production of solubilized enhancins using baculovirus-based expression systems as synergists in novel formulations of baculoviruses with improved efficacy against lepidopteran pests.

To evaluate the feasibility of En3 mass-production, a lab-scale production was carried out both *in vitro*, using Sf9 cultured cells, and *in vivo*, with larvae of *S. exigua* and *T. ni*. Both, tissue culture and live insects could be effectively used for this purpose; enhancins were found in the solubilized fraction of the two systems and could be used in combination with baculovirus OBs. However, production *in vitro* was

more arduous and costly; it involved serial rounds of centrifugations to obtain the solubilized enhancin, which then needed to be purified through 0.2 µm filters to eliminate non-occluded viruses present in the cell medium. Moreover, the final volume of the enhancing substance recovered was low, of 0.5 ml/75 cm^2 flask. In contrast, the *in vivo* system was less labor intensive and yielded higher volumes. It implied the infection of highly susceptible species in their late instars, such as *S. exigua* or *T. ni* (Cohen et al., 2009; Xu et al., 2012), larval harvesting before liquefying, incubation to permit total liquefaction of larvae and purification of the cell soluble fraction by filtration through muslin and centrifugation. The final volume obtained with this system was higher, 10 ml/10 infected larvae.

The enhancing factor present in the soluble fraction increased AcMNPV pathogenicity in heterologous *S. exigua* by 4.2-fold, consistent with previous results (Del Rincón-Castro et al., 2005; Hayakawa et al., 2010; Lepore et al., 1996; Ricarte-Bermejo et al., 2021). Interestingly, the synergistic effect was more intense in the homologous systems. SeMNPV was 15-fold more infective against *S. exigua*, and SpliNPV 11-fold more pathogenic to S*. littoralis* when the En3-containing soluble fractions were added. The specificity of baculoviruses is determined by their ability to enter the cells of susceptible hosts, and then to replicate and produce new infectious virus particles (Rohrmann, 2019; Simón et al., 2004). In this process, the PM is not the only barrier to a productive infection and several mechanisms operate in conferring host cell specificity at different steps. Therefore, disrupting of the PM matrix in homologous hosts eliminates an important physical barrier to highly virulent viruses, magnifying the synergistic effect.

The enhancing effect is clearly instar dependent (Ricarte-Bermejo et al., 2021). Enhancin potentiation is more marked in late instars, usually more resistant to baculovirus infection than their younger counterparts (Bernal et al., 2014; Lei et al., 2019; Wang et al., 1994; Wang and Granados, 2001). In early instars, the PM is generally more permeable, whereas in later instars, the PM is well-formed with low porosity (Erlandson et al., 2019; Lehanne, 1987; Peng et al., 1999). The En3-containing soluble fraction added to the OBs reduced the effective viral concentration against L_4 larvae to a level similar to that for L_2 larvae in *S. exigua*. From a practical point of view this is very remarkable. The overlap of larval generations is very

common in natural populations and an effective crop protection could be better achieved for all pest instars following a single application of a viral insecticide. Due to the lower susceptibility of late instars to baculovirus infections (Bernal et al., 2014), the enhancer substance produced in the present study has attracted interest to control late instars, the principal cause of feeding damage in crops. The inclusion of the enhancer substance could reduce the effective viral concentration to late instars up to 15-fold. This could significantly cut costs associated with the mass production systems.

The main achievement of the present thesis is that the *in vivo* production of solubilized enhancins using baculovirus-based expression systems can be used to improve the efficacy of biological insecticides against lepidopteran pests. The produced enhancer substance can be used in combination with baculoviruses or other *per os* infective pathogens to increase lepidopteran larvae susceptibility to pathogens, reducing the active matter of bioinsecticides and making them commercially competitive with chemicals. In this thesis, a solubilized enhancin substance was produced and purified using an *in vivo* production system. With this method, 10 ml of the enhancer substance were obtained by infecting only 10 larvae. Taking into account that 100 ng/μl is the minimal concentration needed to enhance baculovirus infection, as few as 50 larvae would be necessary to produce the quantity of enhancer substance necessary to treat 1 Ha (1L product).

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CONCLUSIONES

1. Las enhancinas de TnGV han sido las más ensayadas y mostraron una mayor potenciación de las infecciones. Se han identificado tres enhancinas en el genoma de TnGV. Los genes en1, en3 y en4 de TnGV, que tienen marcos abiertos de lectura de 2487 pb, 2706 pb y 2574 pb, y codifican proteínas de 828, 901 y 857 aminoácidos, con un peso molecular de 91, 100 y 95 kDa, respectivamente. Solo la proteína En3 tiene el motivo metaloproteinasa de unión a zinc (HEXXHX (8,28) E), el cual que permite clasificarlas en la familia de las metaloproteasas. Además, el dominio Mucin_bdg también está presente en la proteína En3. Esto sugiere que la proteína En3 podría tener actividad enzimática capaz de degradar la mucina de la PM.

2. La proteína En3 de TnGV expresada en el recombinante de AcMNPV se acumula dentro de las células infectadas Sf9, y no en los OB como en los GV u ODV como en los nucleopoliedrovirus (NPV) y, tras su purificación, y está contenida en la fracción soluble de las células, lo que facilita una producción rentable. La ausencia de dominios de hélice transmembrana impide su envoltura en los ODV, y el hecho de que AcMNPV no posea enhancinas en sus OB hace inviable su oclusión dentro de los OB.

3. La fracción soluble que contiene la En3 es responsable de un aumento de la patogenicidad del AcMNPV en las larvas de *Spodoptera exigua*, y es significativamente más eficaz en el cuarto estadio (hasta 5 veces) que en el segundo estadio (hasta el triple) de las larvas, lo que revela que la producción de las enhancinas solubilizadas mediante un sistema de expresión basado en baculovirus podría ser una herramienta excelente para mejorar los insecticidas basados en baculovirus.

4. Se puede llevar a cabo una producción a escala de laboratorio de la proteína En3 de manera eficaz y rentable utilizando tanto el sistema de producción *in vitro* como *in vivo*, pero el primero es más costoso, ya que implica varias rondas de centrifugación en serie, y es menos eficiente, ya que produce volúmenes finales mucho más bajos que los obtenido in vivo (500 µl frente a 10 ml), lo que demuestra que el uso de larvas para la producción de En3 es más rentable.

5. La fracción soluble que contiene la enhancina En3 aumentó la actividad de AcMNPV en el huésped heterólogo *S. exigua* en 4,2 veces, y en los sistemas homólogos en 15 veces (SeMNPV) y 11 veces (SpliNPV), cuando se usó a concentraciones de 100 ng/µl. No se observaron efectos a concentraciones más bajas, lo que demuestra que esta era la cantidad mínima necesaria para potenciar las infecciones por baculovirus.

6. Utilizando 10 larvas de *T. ni* o *S. exigua*, con el sistema *in vivo*, se obtuvieron 10 ml de sustancia potenciadora a 2000 ng/µl, lo que indica que solo se necesitan cincuenta larvas L5 de *S. exigua* o L6 de *T. ni* para producir suficiente cantidad de sinergista para tratar 1 Ha de cultivos de campo (1 L de volumen de baculovirus formulado).

7. La producción *in vivo* de En3 es una tecnología novedosa para producir una sustancia sinérgica que, en combinación con baculovirus u otros patógenos infecciosos per os, tiene el potencial de aumentar la susceptibilidad de los lepidópteros a los patógenos y, por lo tanto, reducir la materia activa necesaria para controlar las plagas de lepidópteros eficientemente, reduciendo los costes asociados a la producción masiva de bioinsecticidas.

CONCLUSIONS

1. The TnGV enhancins have been the most assayed, and showed the highest enhancement of infections. Three enhancins have been identified in the TnGV genome. The en1, en3, and en4 enhancin genes from TnGV have open reading frames of 2487 bp, 2706 bp, and 2574 bp, and encode proteins of 828, 901, and 857 amino acids with a molecular weight of 91, 100, and 95 kDa, respectively. Only En3 has the zinc metallopeptidase motif (HEXXHX(8,28)E), that allows to classify it into the metalloproteases family. Moreover, the Mucin_bdg super family domain is also present in the En3 protein. This suggest that the En3 protein might have enzymatic activity capable of degrading the mucin of the PM.

2. The TnGV En3 expressed by the AcMNPV recombinant accumulates within infected cells, and not in the OBs as in GVs or ODVs as in nucleopolyhedroviruses (NPVs) and, upon purification, it is contained in the cell soluble fraction, which facilitates production technically and cost-effectively. Probably, the absence of transmembrane helix domains preclude its envelopment in ODVs, and the fact that AcMNPV does not possess enhancins in the OBs make its occlusion within the OBs unfeasible.

3. The soluble fraction is responsible for an increase in AcMNPV pathogenicity in *Spodoptera exigua* larvae, and is particularly more effective in fourth instars (up to 5-fold) than in second instar (up to 3-fold) larvae, which reveals that production of solubilized enhancins using a baculovirus-based expression systems could be an excellent tool to improve baculovirus-based insecticides.

4. A lab-scale production of En3 can be carried out effectively using both *in vitro* and *in vivo*, but the former system is more arduous, as it involves serial centrifugation rounds, and less efficient, as it yields much lower final volumes than those obtained *in vivo* (500 µl vs 10 ml), showing that the use of larvae for the production of En3 is more cost-effective.

5. The enhancin-containing soluble fraction increased AcMNPV activity in the heterologous host *S. exigua* by 4.2-fold, and in the homologous systems by 15-fold (SeMNPV) and 11-fold (SpliNPV), when it was used at 100 ng/µl, but no effects were observed at lower concentrations, demonstrating this was the minimum amount needed to enhance baculovirus infections.

6. Using the *in vivo* system, 10 ml of enhancer substance at 2000 ng/µl was obtained using 10 *T. ni* or *S. exigua* larvae, indicating that only fifty L_5 *S. exigua* or L_6 *T. ni* larvae are needed to produce enough amount of synergist to treat 1 Ha of field crops (1 L volume of formulated baculovirus).

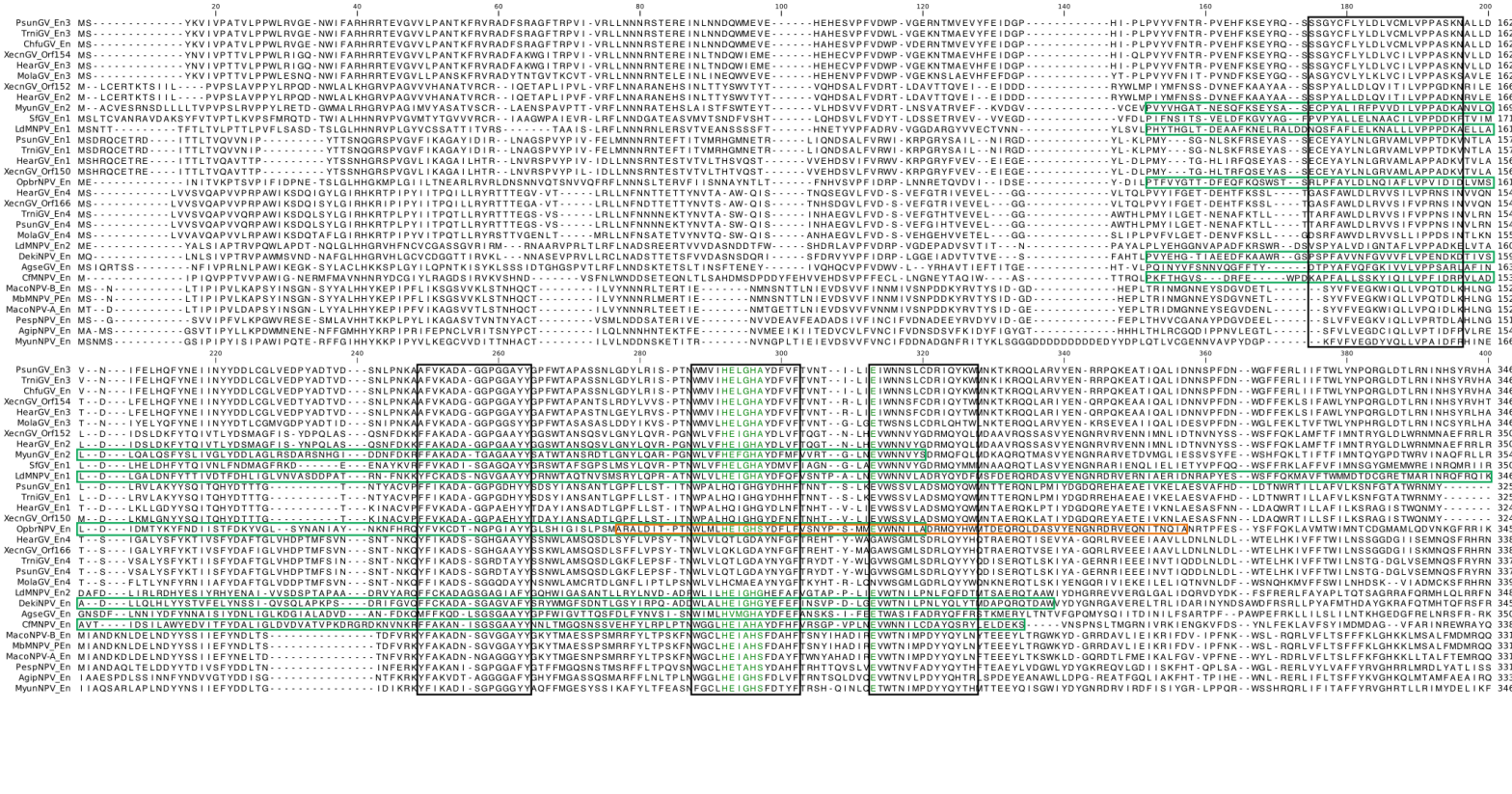
7. The *in vivo* production of En3 is a novel technology to produce a synergistic substance that, in combination with baculoviruses or other *per os* infective pathogens, has the potential to increase lepidopteran susceptibility to pathogens and hence reduce the active matter necessary to control lepidopteran pests efficiently, reducing the costs associated with the massive production of bioinsecticides.

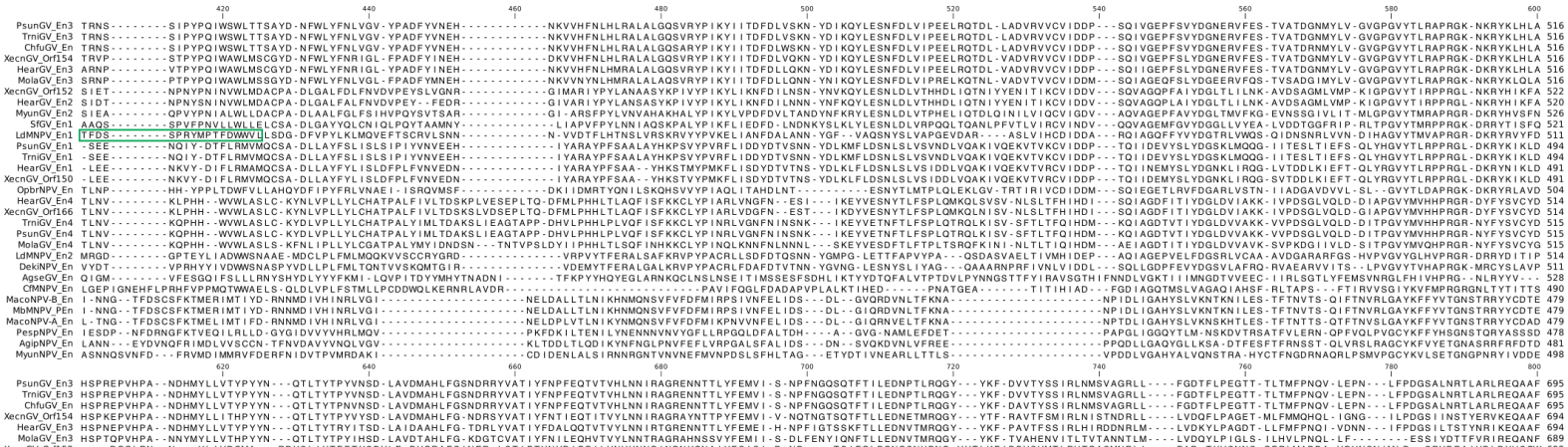
LIST OF PUBLICATIONS

Ricarte-Bermejo, A., Simón, O., Fernández, A.B., Williams, T., Caballero, P. 2021. Bacmid expression of granulovirus enhancin En3 accumulates in cell soluble fraction to potentiate nucleopolyhedrovirus infection. Viruses 13, 1233.

Suplemental material

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1. Global alignment of enhancin proteins. The HEXXHX(8,28)E motif is represented with light green residues. The transmembrane helices are represented in red residues. The Peptidase_M60 Superfamily, the SslE_AcfD_Zn_LP and Mucin_bdg super family domains are shown in green, orange, and blue boxes, respectively.





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